THE SEPARATION OF HISTONE COMPONENTS

A Thesis presented for the

Degree of Doctor of Philosophy

by

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GENERAL INTRODUCTION

The basic protein histone was first found by Kossel (1884) in goose erythrocyte nuclei. In 1894 Lilienfield announced its existence in thymus gland, and ten years later Ackermann discovered that it also occurs in fowl erythrocyte nuclei. In 1928 Kossel showed that it replaces protamine in the spermatozoa of some fish.

More recently, many papers have been published describing the occurrence and distribution of histones (Mirsky and Pollister, 1942; Stedman and Stedman, 1944, 1947; Cruft, Mauritzen and Stedman, 1957a; Mirsky and Osawa, 1961; Phillips, 1962), and now it is generally accepted that they exist in the somatic cell nuclei of all vertebrates. They are present in the spermatozoa of some vertebrates although the simpler protamines occur in the spermatozoa of certain fish and the chicken.

As far as the invertebrates are concerned, the literature lacks extended studies, although they have been shown to exist in the developing embryo of Helix Aspersa by using cytological staining and in the spermatozoa of some echinoderms.

In plants, histones have been isolated from some species and by cytological techniques shown to exist in some others.

All the above mentioned refers to the occurrence of histones or basic proteins in the cell nucleus. However Crampton and Petermann (1959) announced the presence of basic proteins in the microsomes of rat liver. Analytical results showed that these are similar to histones although considerable differences exist in amino acid and N-terminal group analyses. Butler, Cohn and Simpson (1960) investigated the proteins of rat liver ribosomes and after some purifications obtained a protein as basic as histone but differing in free electrophoretic pattern, alanine content, and in electrophoresis in starch gel. Thus although the existence of cytoplasmic basic proteins is more or less established, the relationship, if any, between these and histones, which are of nuclear origin, is not clear.

In order to clarify the exact meaning of the term histone, reference is made to the definition given by Cruft et al. (1957a). They suggested that the name histone "should be reserved exclusively for basic proteins, other

than protamines, which occur in the cell nucleus". This definition was to be justified by the discovery of histone-like proteins in the ribosomes. More recently, in the First World Conference on Histones, Murray (1964) suggested that the term histone should be applied to "basic proteins that at some time are associated with DNA". The meaning of "histone" was, according to Murray, broadened in order to include protamines, as the difference between protamines and histones is not greater than that between two histone fractions with widely differing properties.

In the course of this Thesis the definition given by Cruft et al. (1957a) will be followed throughout.

The histones are extracted either from whole cells or from isolated nuclei. Although the reasons for preferring the second procedure are obvious, some workers continue to use whole homogenized tissue. It must be stressed that

there is no general technique to be followed, as each organ presents difficulties of its own.

The most commonly used methods are the extraction of whole tissues or isolated nuclei with either dilute acid, which extracts the histones, or with a molar solution of sodium chloride which extracts the nucleoprotein complex. Stedman and Stedman (1951) used dilute sulphuric acid to extract histones from isolated nuclei, and precipitated them with ethanol. Bakay, Kolb and Toennies (1955); Luck, Cook, Eldredge, Haley, Kupke and Rasmussen (1956); Hirschbein and Khouvine (1957); Ui (1957); Bijvoet (1957), and many others, used dilute hydrochloric acid for the extraction of histones and they generally lyophilized the histone extract after dialysis against distilled water. Bakay (1957) shook a sodium chloride extract (containing nucleoprotein) with chloroform and octanol at pH 11 to precipitate the histones. But it is doubtful whether this procedure precipitates all the lysine-rich

histone fraction, (Phillips, 1962). Whatever the technique applied, the product isolated consists of 'crude' or unfractionated histone. This histone shows complex patterns in chromatographic and electrophoretic analyses, and many attempts have been made to obtain pure histone fractions. The experiments of Stedman et al. (1951) were the first to show the heterogeneity of these proteins. While attempting to purify histone, isolated from calf thymus gland, by repeated ethanol precipitation, they obtained two fractions: one a lysine-rich protein in which lysine accounted for 40% of the nitrogen and arginine for 5%, and the other an arginine-rich protein in which arginine accounted for 28% of the nitrogen and lysine for 16%. The first was more soluble and easily extractable and the second more readily precipitated. These fractions were also obtained by Davison and Butler (1954), and Daly and Mirsky (1955). The ethanol fractional precipitation was also employed by Gregoire and Limozin (1954); Ui (1957); Bijvoet (1957) and Cruft et al. (1957a).

The precipitation of histones at their isoelectric points was used by Davison, James, Shooter and Butler (1954); Daly et al. (1955) and Davison and Shooter (1956). Cruft et al. (1957a) applied ethanol precipitation at the isoelectric point together with rising ionic strength, and obtained three fractions.

The amino acid analysis of the various fractions are given in the Appendix, which also shows the nomenclature used by the various workers.

The methods used for the isolation of nuclei can be divided into two parts: firstly the lysis of the cells by acids, and secondly the homogenization of the tissues in various solvents.

The most common procedure for the lysis of cells is based on the observation by Lewis (1918) that the nuclear membrane becomes more distinct at a pH between 3.8 and 4.6. Crossman (1937) first applied low pH for the expulsion of the nucleus by causing the cell

to burst in 5% citric acid. Stedman et al. (1951) used 4% acetic acid successfully for the same purpose.

Media which have been used for the disruption of the cells by homogenization, include saline-calcium chloride (Dounce, 1952) a wide range of concentrations of sucrose ranging from 0.25 M (Schneider, 1948) to 2.2 M (Chauveau, 1952), sucrose-calcium chloride (Hogeboom, Schneider and Striebich, 1952), sucrose-magnesium chloride (Stirpe and Aldridge, 1961), glycerol (Schneider, 1955) and ethylene glycol (Luck et al. 1956).

The comparison of the results given by the various methods is rather difficult, as the criteria for 'pure' nuclei are neither strict, nor generally accepted. However whatever method is used should give nuclei free of cytoplasmic material and morphologically intact. In the case of histones most of the work is done with products extracted from calf thymus nuclei and the disruption of these cells with acetic acid was thoroughly investigated by Stedman et al. (1951) and the results were excellent.

This method was thus used throughout this investigation both for calf thymus and rat liver, so that the results could be compared.

Free electrophoresis was first used on histones by Cruft (1953), and showed three boundaries with calf thymus histone. The same pattern was obtained by Butler, Davison, James and Shooter (1954); Davison *et al.* (1954) and Gregoire *et al.* (1954). Cruft *et al.* (1957a) extended their studies by using histones from about a dozen sources. Most of these showed three groups at pH 6 to 7.6, which were named by Cruft and co-workers α, β and γ according to their mobility. The α fraction in an electrophoretic run in a trough of starch grains was further resolved into three fractions, namely α_1, α_2 and α_3 according to their mobility (Cruft, Hindley, Mauritzen and Stedman, 1957b). The amino acid analysis of these fractions is given in the Appendix.

The electrophoresis of histones on paper was attempted by Luck et al. (1956), and Hnilica (1959). Their results were rather poor as they obtained patterns showing only two bands. More recently Hnilica, Gregusova and Thurso (1960) improved the method by running the histones in pH 4.8 buffer made 8 M with respect to urea, and by also keeping the temperature low (2°C) they showed five bands on bromphenol staining.

A major breakthrough in electrophoretic analysis occurred when Neelin and Connell (1959) applied starch gel electrophoresis (Smithies, 1955) to histones. They used chicken erythrocyte histone at pH 4.1 to 4.9 and showed sixteen positively charged bands. By cutting and eluting the bands off the gel, they showed that the eluants migrated as single bands and occupied exactly the same positions on the gel as they originally had. They claimed that this supports the argument that the bands were not artifacts of the method. Later Neelin and Neelin (1960) examined calf thymus histones by the same technique and obtained twenty-two bands, of which they thought sixteen were due to

native histone. Their results were confirmed by other workers and for the first time it became obvious that histone constitutes an extremely complex protein group.

An improvement in zone electrophoretic analysis of histones was the use of polyacrylamide gel by Cruft (1962). The patterns obtained were quite different from that with starch gel, but the resolution was much sharper and the order of migration closer to that of free electrophoresis. Similar results on polyacrylamide gels were obtained by McAllister, Wan and Irvin (1963), and Drieger, Johnson and Marks (1963).

The electrophoresis of histones in the above named gel will be discussed more extensively in Chapter One.

The first attempt to fractionate histones using purely chromatographic techniques was made by Crampton, Moore and Stein (1955). They used the ion exchange resin Amberlite IRC-50 in its barium salt form, and eluted the histones

with a gradient of barium acetate at pH 6.7. This yielded two peaks, namely A and B, which constituted 75% of the whole histone used for the fraction. Fraction A was a very lysine-rich histone and B a slightly lysine-rich one. Fraction B was later claimed by the same workers to be heterogeneous (Crampton et al. 1957). The above results were confirmed by Hindley (1957). Luck et al. (1956) and Luck, Rasmussen, Satake and Tsvetekov (1958) used the same resin, IRC-50, but in its sodium form and eluted with a gradient of guanidinum chloride. This resulted in a quantitative recovery of the histone applied to the column, in several peaks. The first two peaks (which were overlapping) were due to lysine-rich histones. The next peak was composed of slightly lysine-rich proteins, and finally the last double peak contained the arginine-rich histones. In 1959, Neelin and Butler, by using IRC-50 in the sodium form and eluting with sodium chloride solutions in concentrations up to 3 M, recovered 80% of their histone in one lysine-rich fraction and three arginine-rich fractions.

The use of carboxymethylcellulose (CM-cellulose) was initiated by Davison et al. (1956) and Davison (1957). They fractionated calf thymus histone using a sodium chloride gradient in the pH range 3 to 6.5. This yielded a quantitative elution of histone in two main fractions, the first being a very lysine-rich one. Phillips and Johns (1959) used CM-cellulose and eluted the histones with acid in order to eliminate aggregation effects. With 0.05 N hydrochloric acid 'small acidic contaminants' were eluted. With 0.01 N hydrochloric acid 51 - 67% of the histone was eluted and a fraction named F1 was obtained, consisting of a 'moderately lysine-rich histone' (Lys./Arg. : 2). Stepping the hydrochloric acid concentration up to 0.02 N a second fraction was eluted, named F2, which had a lys./arg. ratio of 1.3. It becomes obvious that the very lysine-rich histones do not appear anywhere in this fractionation. It was later shown by Johns, Phillips, Simson and Butler (1960) that this fraction can be eluted by acetate buffer pH 4.2, before the elution with acid started. Similar results

were obtained by Sautiere (1960), and Hirschbein and Rosencwajg (1960). However, when Davis and Busch (1959) tried to elute the protein from their CM-cellulose columns with pH 4.2 acetate buffer, they found they could not do so. The elution could be effected with a formic acid gradient from 1 N to 8 N in the pH range 2.6 - 1.6.

A modification of the above method of Johns et al. (1960) was mentioned in Johns' Thesis (1963), and consisted of the addition of sodium chloride in the acetate buffer, for the elution of the very lysine-rich histones. This method will be further discussed in Chapter Two.

Of particular interest is the use of gel filtration for the fractionation of histones, because of the mild conditions used. It was first applied by Cruft (1961). He used Sephadex G-75 and calf thymus histone dissolved in 0.02 N hydrochloric acid. The elution carried out with the same solvent yielded four

peaks containing different histone fractions. The method was extensively investigated by Muecke (1962). In one instance he obtained six distinct fractions with different electrophoretic patterns when run in starch gel. It was further applied by Hnilica and Bess (1964) and Hnilica (1965), for the fractionation of arginine-rich histones of calf thymus, and Hnilica (1964) also applied it to the separation of lysine-rich histones from chicken erythrocytes.

Phillips (1957) and Hindley (1957) attempted to fractionate histones on Alumina columns. The results were by no means promising and for that reason the method was not pursued any further.

The extreme heterogeneity of histones was attributed by many workers to the presence of proteolytic enzymes acting either during their preparation or storage.

In 1952, Maver, Greco, Löwtrop and Dalton announced the presence of intracellular cathepsins in thymus gland, and Butler et al. (1954) suspected that these enzymes contributed to the heterogeneity of histones extracted from nucleoprotein. Evidence for proteolytic activity in histone preparations was also given by Crampton et al. (1957) and Davison (1957) and chymotrypsin activity in histones was reported by Phillips et al. (1959).

The catheptic activity of histones was demonstrated with haemoglobin as a substrate though, according to Phillips (1962), it could not be detected with histones extracted with acid. It was also announced (Phillips, 1962) that the chymotrypsin activity of the histones was suppressed by diisopropylfluorophosphate or by lowering the pH to 3.

All the experiments just mentioned were done with histones extracted with neutral 1 M sodium chloride, either from whole tissues or from isolated nuclei known to be contaminated with cytoplasmic material.

Cruft, Mauritzen and Stedman (1958b) considered the reports dealing with proteolytic activity of histones, and they stressed that they have never observed any evidence to support it. It is worth mentioning that they have always extracted histone, from isolated nuclei, with acid.

Leslie (1961) demonstrated ribonuclease activity in histones and ribosomal basic proteins. This activity was rather low, and later Martin, England, Turkington and Leslie (1963) showed that it was present only in a small portion of the basic proteins.

The tendency of histones to aggregate and give rise to larger molecules was investigated by Cruft, Mauritzen and Stedman (1954); Cruft et al. (1957a); Ui (1956, 1957) and Cruft et al. (1958a, 1958b). From their work done with β and γ-histones, arginine-rich and slightly lysine-rich respectively, it was found that the aggregation of the β fraction is promoted by an increase in pH, ionic strength, anion valency,

concentration, temperature and time. That of γ-histone is suppressed by an increase in ionic strength. The presence of other histones or of the trivalent cations Lanthanum and Aluminium strongly suppresses the aggregation of the β-histone.

Throughout the experimental work an attempt has always been made to keep as close as possible to conditions suppressing aggregation.

The association of histones with chromosomes has given rise to many speculations concerning the functions of these proteins. Stedman and Stedman (1950, 1951) after extensive purification studies on β-histones (arginine-rich) published their theory on cell-specificity of histones. According to it, the various histones combine with those parts of DNA not required for the production of a given type of cell, and only the 'free' part of DNA determines the character of the cell. In favour of this theory was the fact that in normal resting cells histones comprise 1.6% to

24% of the total dry weight of the nucleus, while in embryonic cells and tumours comprise 1.6% to 3% of the total dry weight of the nucleus. Hence the high histone content prevents a cell undergoing mitosis. Evidence supporting this theory was obtained by Cruft et al. (1957a) who studied the β-histones from thymus gland and liver cells of the calf. They found a small difference in electrophoretic mobilities, confirmed by amino acid analysis. These studies were extended to the β-histones from many other bovine and chicken tissues (Mauritzen and Stedman, 1959, 1960).

Bonner, Huang and Gilden (1963) studied the chromatin dependent synthesis of a cell specific pea seedling protein, and obtained evidence in favour of the Stedmans' hypothesis.

Further evidence was presented by Hnilica (1964) who isolated an unusual lysine-rich histone from chicken erythrocytes.

In a publication by Johns, Phillips, Simson and Butler (1961), the gene suppressor theory of the Stedmans' is contended on the basis of the similarity of amino acid analyses of the

three main histone groups extracted from a variety of ox and rat tissues. All these analyses were made with fractions shown to be composite by starch gel electrophoresis, and the procedure applied for their extraction was found not to be applicable to all tissues examined; while the extraction and fractionation procedure applied by Stedman and his colleagues was reproducible and was applicable in all cases.

It has been suggested by Butler (1956) that histones may be involved in mitosis, possibly by affecting the separation of the chromatids, or having a purely structural function by linking the DNA molecules into the chromosome. This seems unlikely as the morphology of the chromosome remains intact when histone is removed by the Feulgen staining technique.

The hypothesis that histones have as their only function the neutralization of the acid groups of DNA and so prevent the DNA from associating with other proteins or metal ions put forward by Vendrely, Knobloch, Mazen and Vendrely (1960) is sound as far as the

equivalence of phosphate groups on the DNA molecule and basic groups on histones is concerned. But this theory does not take into account the extreme diversity of histones nor their relatively high turnover rate (reviewed by Phillips, 1962).

A more recent suggestion for the function of histones came from Leslie (1961). He put forward the idea that histones may control some cell differentiation processes by stabilizing certain RNA templates and eliminating some others, both in the nucleus and in the cytoplasm. He based his conclusions on the fact that histones show some ribonuclease activity.

In view of what has been mentioned it is premature to draw any conclusions on the role of histones. The only thing that is beyond any doubt is their complexity. So far all attempts to fractionate histones and obtain an analytically pure histone have failed. Unless a technique for the fractionation of these proteins is developed no one of these theories can be proved or rejected.

These considerations were the prompting ideas behind this work, throughout which, the final aim has always been the development of a technique that might yield results illuminating this rather obscure problem.

CHAPTER ONE

POLYACRYLAMIDE GEL ELECTROPHORESIS OF HISTONES

PART I

APPLICATION TO CALF THYMUS AND RAT LIVER HISTONES

Introduction

In 1962 H.J. Cruft published his work on the electrophoresis of calf thymus histones in polyacrylamide gel.

The previously available method, starch gel electrophoresis, of Neelin and Connell (1959), although it showed for the first time the extreme complexity of histones, was unsatisfactory in so far that the patterns obtained bore little resemblance to those of moving boundary electrophoresis. In free electrophoresis the α-histones (lysine-rich) show the highest mobility, followed by the β-histones (arginine-rich) and the γ-histones. In starch gel electrophoresis the order of migration of the groups is different. In addition to this, when fractions are run alongside unfractionated histone, the bands do not occupy the same positions in the gel, (Muecke, 1962). This irregularity is most probably due to the free

carboxyl groups present in starch gel, which interact with the histones and slow them down.

In polyacrylamide gel these carboxyl groups are not present and it might be expected that the patterns obtained would correspond to the histone's macromolecular properties. With this gel, after staining with Amido Black 10B, Cruft observed that the a-histones ran ahead of the rest and separated into four turquoise bands. Behind those were two main deeply stained blue-black bands, and between these and the origin five faint bands. At the origin there was usually a trace of material that did not penetrate the gel due, it is thought, to aggregation. The bands were much sharper than in starch gel electrophoresis, and their reproducibility found to be excellent. Furthermore the pore size of the acrylamide gels can be varied by varying the acrylamide concentration, and it was noticed that by reducing it no aggregated material was left at the origin, although the resolution was now poorer.

Acrylamide gels can be prepared over a wide range of pH values (2 - 10) and lanthanum salts, known to suppress histone aggregation, can be incorporated (Cruft et al. 1958).

Some preliminary experiments were therefore undertaken first to duplicate and then to extend Cruft's work.

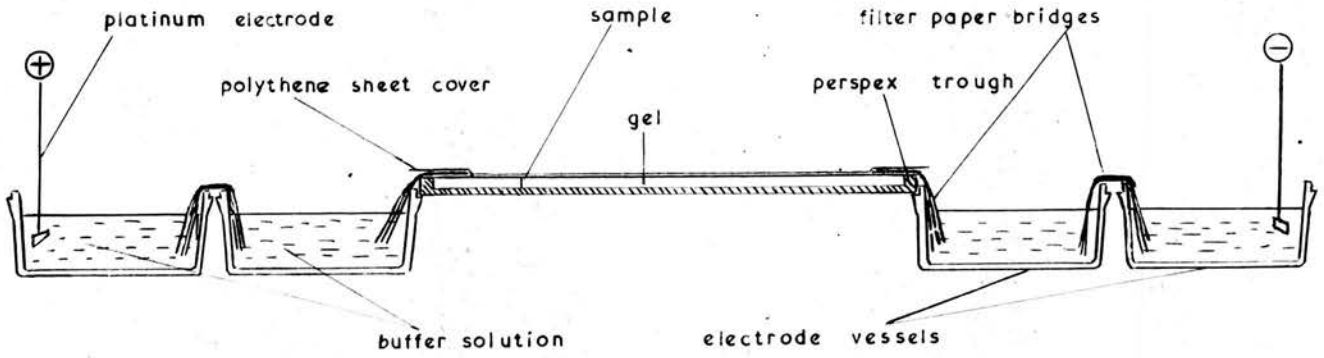
Experimental

Preparation of polyacrylamide gel

The gels were prepared according to the method of Cruft (1962) which is a modification of the original work of Raymond and Wang (1960).

Ten grams of acrylamide (Eastman Kodak, Ltd.) and 200 mg. of N,N'-methylenebisacrylamide (L. Light & Co. Ltd.) were dissolved in 100 ml. of Lanthanum-Acetate buffer pH 4.9 (0.02 M acetate buffer pH 4.2 made up to 0.02 M in Lanthanum - Acetate and pH adjusted if needed with glacial acetic acid to 4.9). To the above buffer were added with gentle stirring 1 ml. of a 10% (v/v) solution of N,N,N',N'-tetramethylethylenediamine (L. Light & Co. Ltd.) and 1 ml. of freshly prepared 10% (w/v) aqueous ammonium persulphate (B.D.H.). This solution was then subjected to water pump vacuum for about one minute, in order to remove dissolved air, and poured into perspex troughs. A perspex lid was placed on the top, care being taken not to include any air bubbles, the excess liquid expelled, and a weight put on the top. The whole was left

Fig I ELECTROPHORESIS SET-UP

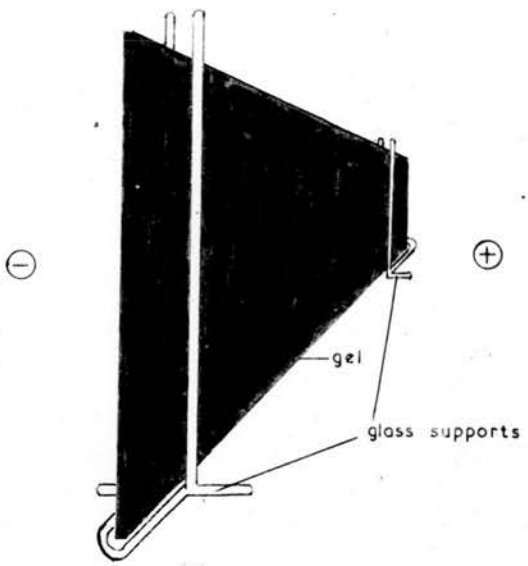


undisturbed for a few hours until polymerization had occurred. The gels were generally used the following day. The dimensions of the trough varied except for the depth which was always 3 mm.

Electrophoretic set up

The sample was applied on a strip of filter paper about 2.5 mm. wide and introduced in slits made with a razor blade. The electrode vessels were filled with 0.02 M acetate buffer pH 4.2 except for the one next to the gel on the anode side which was filled with Lanthanum-Acetate buffer pH 4.9. The connections between gel and vessels and between vessels were established with five layers of filter paper. The voltage was applied between platinum electrodes and measured directly on the gel surface with an electronic voltmeter. During the electrophoresis the gel surface was kept covered with a polythene sheet to prevent evaporation. No cooling was applied (Fig. 1). The voltage through the gel was of the order of 4 v/cm. and electrophoresis usually lasted 5hr.

Fig. 2 DESTAINING OF THE GEL



Staining and destaining of the gels

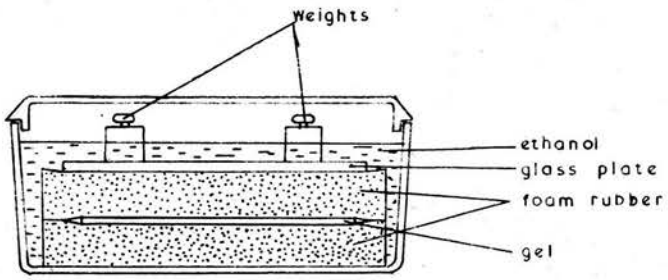
The gels were stained for 10 min. with 0.5% (w/v) solution of Amido Black 10B in methanol-water-acetic acid 5:5:1, and destained in the same solvent either by frequent changes of the bath or by electrophoresis. For electrophoretic destaining the gels were supported vertically with their longer edge on the bottom of a vessel containing the methanol-water-acetic acid mixture, and held in position with specially made glass supports (Fig. 2). 300 Volts were applied across the vessel and the destaining was over in about 10 min.

Storage of the gels

The gels were kept in the water-methanol-acetic acid mixture. In this solution a slight swelling of the gels occur, but they do not lose their shape. The bands fade considerably however, and the faint ones cannot be seen after a few weeks.

The gels can be dried to a thin film on a glass plate but they often shrink unevenly and break. These difficulties were overcome to a

Fig. 3 DEHYDRATION OF THE GEL



certain extent by adding 1% glycerol to the water-methanol-acetic acid mixture, and allowing the gels to remain in this solution for 24 hr. before drying. The results, however, were found to be inconsistent and a dehydrating technique using ethanol was applied whenever it was desirable to keep the gels.

The gels were placed in a vessel between two 'foam rubber' sheets. A piece of glass having approximately the same dimensions as the gels was placed on top, and the whole was kept under pressure with two weights placed on top of the glass plate. The vessel was then filled with ethanol and left undisturbed for one day (Fig. 3). The gels so treated became solid and white and the visibility of the bands was much improved against the white background.

An uneven shrinkage of the gels occurs, but its extent is limited enough not to cause any confusion or distortion of the results.

Electrophoresis of calf thymus histones

Unfractionated histone

The histones used for the polyacrylamide gel electrophoresis were extracted according to

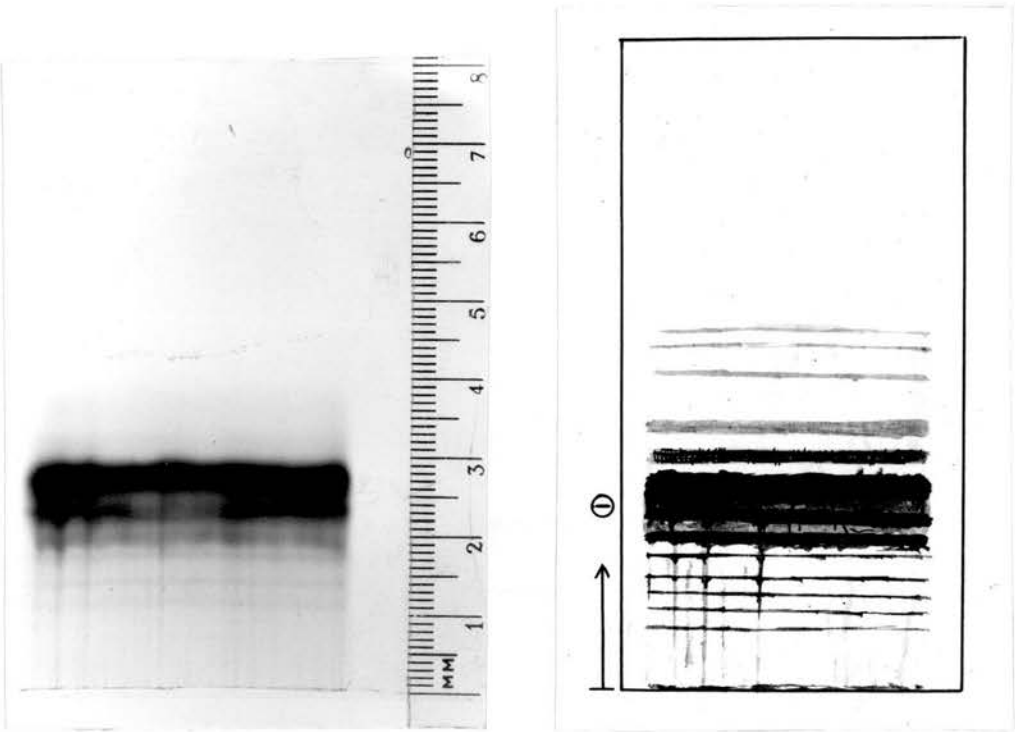


Fig. 4. POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS WHOLE HISTONE. LANTHANUM-ACETATE BUFFER PH: 4.9

the method of Stedman et al. (1951). The details of this method are given in Chapter Two. The whole (unfractionated) histone was submitted to polacrylamide gel electrophoresis. The result of such a run is shown in Fig. 4. This run was done with a sample of 4% (w/v) whole histone dissolved in Lanthanum-Acetate buffer pH 4.9 and acidified with a few drops of glacial acetic acid to pH 3 and the running time was 5 hr. The preparation of the sample took place 24 hr. before the actual run, as it was noticed that running a sample immediately after its preparation gave rise to much aggregated material in the starting line. As the photographic reproduction of the stained gels is very poor, due to the wide differences in the contrast of the bands, it was thought necessary to introduce drawings which can give a better representation of the original. This has been done in most of the cases. In this electrophoretic run 13 bands in all are visible. The first five (fastest migrating) bands belong to the α (lysine-rich) histones; the very dark one in the middle of the gel together with the

next behind it are the γ-histones, and the β-histones (arginine-rich) are the remaining five bands. The fraction to which each band corresponds was established by simultaneous running of the unfractionated histone with its fractions. The resolution in the middle of the gel is quite poor as many bands show approximately the same electrophoretic mobilities. Better resolution is shown when the histone fractions are run individually.

Many attempts have been made to improve on the electrophoretic pattern by varying the lanthanum acetate concentration, the protein concentration in the sample, the acrylamide concentration in the gel, and the time. As lanthanum salts are known to suppress the aggregation of histones (Cruft et al. 1958a) their concentration in the acetate buffer was stepped up from 0.02 M to 1 M. In no case did this make an obvious difference. It seems that the effect of lanthanum salts reaches the peak of its activity at a concentration of 0.02 M and further addition has no effect at all.

The variation of protein concentration in the sample applied to the gel, gave rise to electrophoretic patterns differing widely in the number of bands and their staining intensity. If the protein was reduced most of the faint α-bands could hardly be seen, and the β-group too showed fewer bands when the concentration dropped below 2%. The intense γ-bands did not show much difference with variations in protein concentration of the sample. By increasing the protein content of the sample the pattern became darker and at about 8% there was no definition on the region of the β and γ-bands.

The concentration of the acrylamide was more critical than any of the other factors. By reducing it to 5 g. the gels became viscous and very difficult to handle. Runs made in these gels showed no sharp definition although most of the aggregated material usually present in the starting line disappeared. On the other hand raising the concentration of acrylamide produced stiffer gels in which the

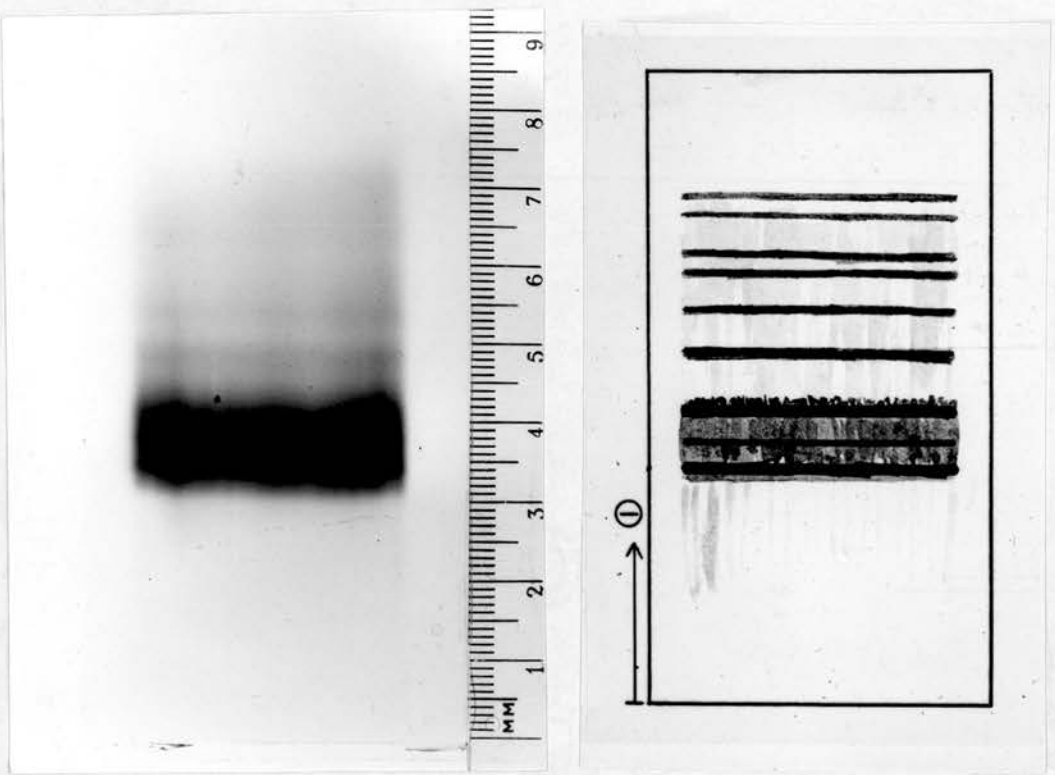


Fig. 5 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS a-HISTONE LANTHANUM-ACETATE BUFFER PH: 4.9

distance migrated was shortened, the bands became narrower and the distance between the bands shorter. The material that remained in the starting line increased enormously over the amount of non-migrating material found in up to 10 g. polyacrylamide gels, and most of the β-bands disappeared, obviously not being able to penetrate the gel structure.

By lengthening the running time it was found that the bands migrated further, but they lost much of their sharpness due to diffusion, and thus no advantage was gained.

Electrophoresis of histone fractions

The histone fractions used for the electrophoresis were prepared according to Cruft et al. (1958a). The method is described in Chapter Two.

α-Histones

The electrophoresis of α-histones was performed in exactly the same way as that described for the whole histone. Fig. 5 shows a typical pattern. A drawing has been inserted in order to show the detail that is not visible in the

photograph. This run was done with a 4% α-histone sample in lanthanum-acetate buffer acidified with glacial acetic acid to pH 3. The time was 4 hr. at 4 v/cm. Nine bands in all are visible, of which the fastest six are the characteristic turquoise coloured bands. No aggregated material is left behind in the starting line, and this is due to the low molecular weight of the components of this histone group.

The attempts to improve resolution for the unfractionated histone, were repeated with the α-histones although this fraction is the one that usually shows no aggregation at all, even in the absence of lanthanum salts, and as a rule, the nine bands shown in Fig. 5 are visible in every run. The distortion due to material left trailing behind is negligible, compared with that shown by the other fractions.

β-Histones

This fraction is the one most susceptible to aggregation and special care has to be taken to minimize this. The usual 4% protein

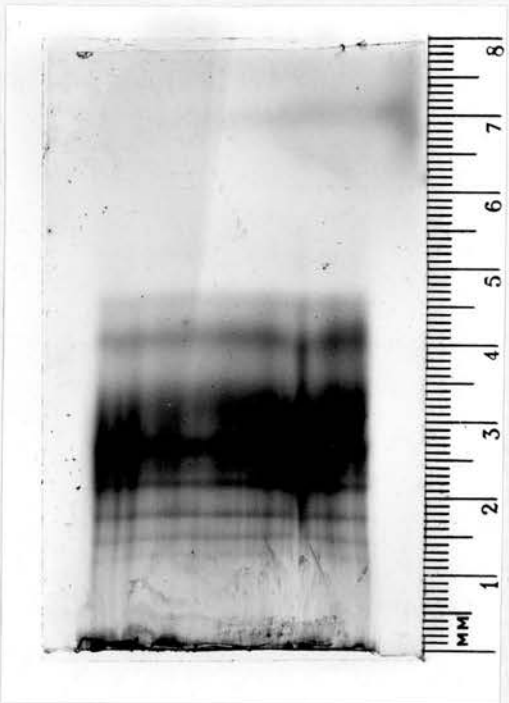


Fig. 6 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS β-HISTONE. LANTHANUM-ACETATE BUFFER PH: 4.9

concentration of the sample applied for electrophoresis gives rise to patterns in which five to seven bands are visible compared with nine in non-aggregated sample. It is known that the aggregation of this histone group is promoted by increasing the protein concentration (Cruft et al. 1958b). By reducing the concentration to 2%, using, as usual lanthanum-acetate buffer, and lowering the pH to 3 with acetic acid, patterns like the one shown in Fig. 6 are obtained. In this, which was run for 5 hr. at 4 v/cm., nine bands are clearly visible although the actual contrast is somewhat less than that suggested by the photograph. The 'trailing'effects are very noticeable as also is the aggregated material in the starting line, which does not penetrate the gel.

Attempts to improve the pattern yielded by the β-histone by changing the acrylamide concentration, by reducing the protein in the sample, and by running the electrophoresis at 1 - 5 v/cm. for various periods of time did not succeed.

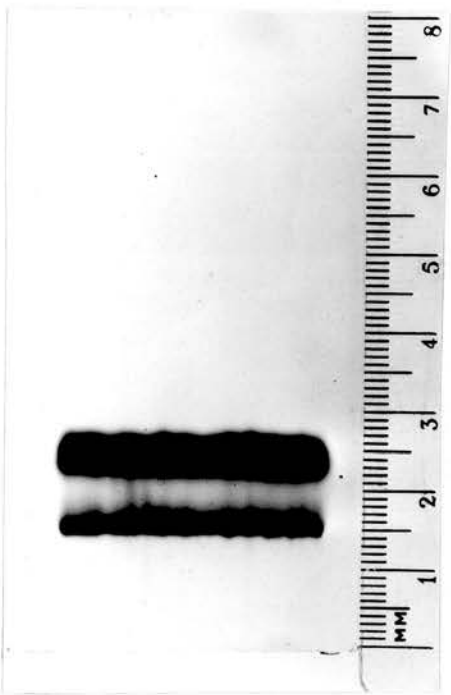


Fig. 7 POLYACRYLAMIDE GEL ELECTROPHORESIS
OF CALF THYMUS γ-HISTONE
LANTHANUM-ACETATE BUFFER PH: 4.9

γ-Histone

A typical electrophoretic pattern of the γ-histone is shown in Fig. 7. The electrophoresis was carried out exactly as described for the other fractions. A 4% sample on lanthanum-acetate buffer was acidified with acetic acid to pH 3 and run for 5 hr. at 4 v/cm. The bands are very heavily stained blue, and the photograph of the pattern clear. The wider band which migrates ahead is a double one, but the distance between the two components is extremely small and just visible with the naked eye.

By changing the concentrations of protein in the sample or of acrylamide in the gel the pattern could not be improved. Here there is no aggregated material in the starting line for although the γ-histone has a tendency to aggregate, it does not do so to the extent exhibited by the β-histone.

Run of unfractionated histone simultaneously with the three fractions

This run is shown in Fig. 8. A sketch is inserted with all the details of the gels, as

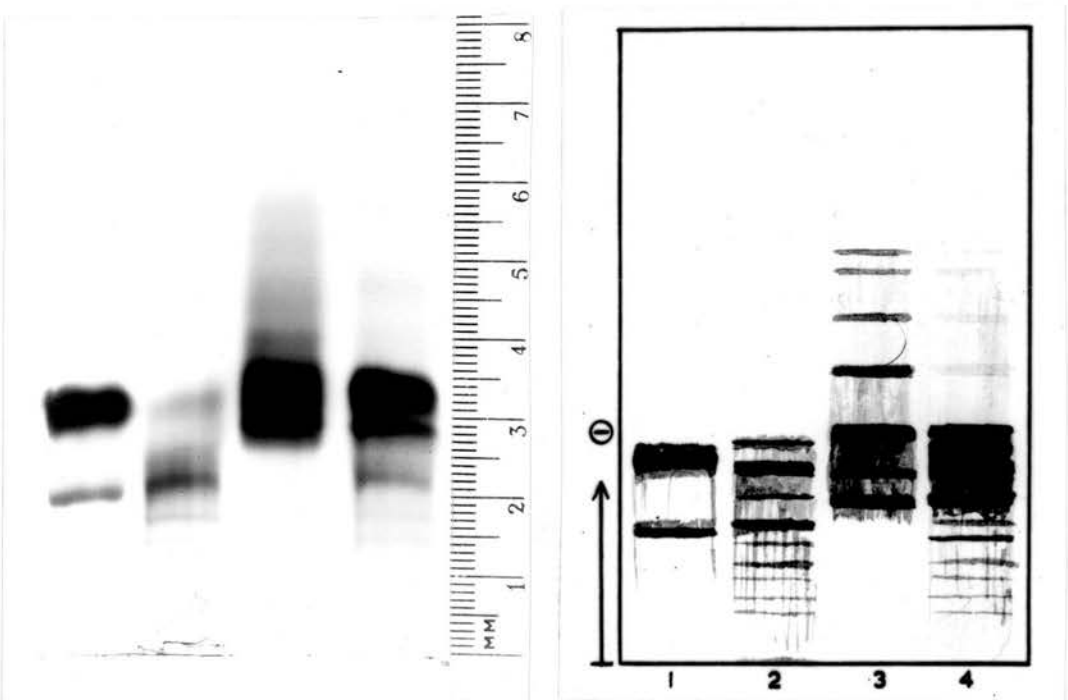


Fig. 8 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS HISTONE FRACTIONS.

1 γ -HISTONE

2 β- HISTONE

3 α -HISTONE

4 UNFRACTIONATED HISTONE

LANTHANUM-ACETATE BUFFER PH: 4.9

the photograph lacks definition. The samples applied were 4% in lanthanum-acetate buffer, acidified with acetic acid, except for that of the β-histone which was 2%.

The relative positions of the bands of the various fractions can be compared with those of the unfractionated histone.

Electrophoresis of rat liver histones

The techniques used were in all cases as described for the calf thymus histones. The patterns obtained are different from those of calf thymus and they usually lack clarity and sharp definition. The histones from rat liver were found to aggregate much more readily than calf thymus ones, and to be more difficult to dissolve.

Electrophoresis of unfractionated rat liver histone

Normal rat liver

The definition of normal applied to 'rat liver' is used in order to distinguish it from 'regenerating rat liver'.

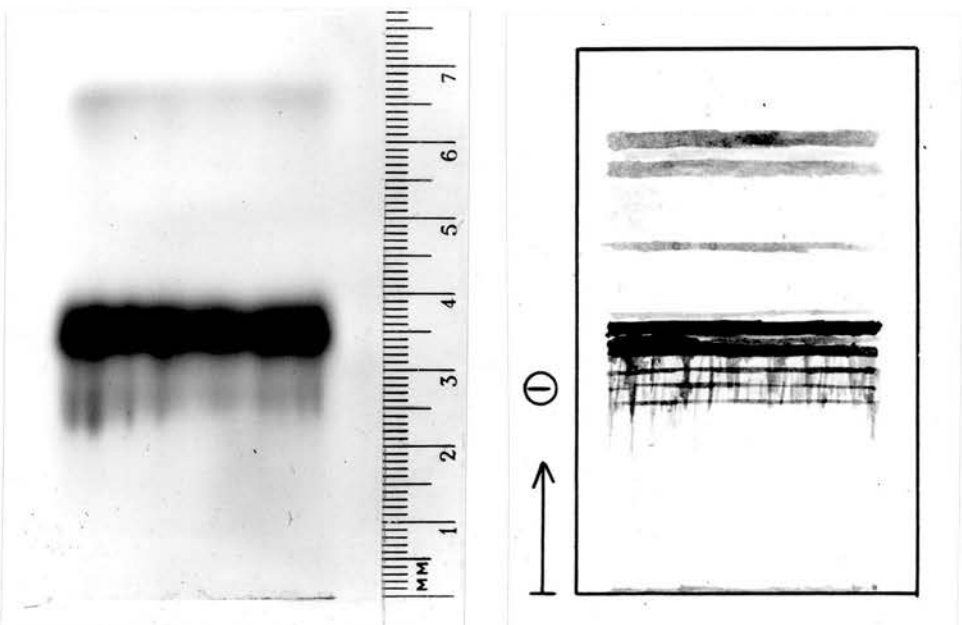


Fig. 9. POLYACRYLAMIDE GEL ELECTROPHORESIS OF RAT LIVER WHOLE HISTONE LANTHANUM-ACETATE BUFFER PH: 4·9

The electrophoretic pattern yielded by whole histone from normal rat liver is shown in Fig. 9. Again it has been found necessary to insert a sketch so that the details of the gel can be appreciated. This pattern was obtained with a 4% sample prepared as before, in an electrophoretic run that lasted 4 hr. at 4 v/cm. The number of bands is less than that seen in similar electrophoresis of calf thymus histones. The most striking difference is in the faster migrating fractions (α-histones). A lot of diffusion takes place in this region, and the number of distinct bands is fewer, and less well defined than that from calf thymus.

Regenerating rat liver

Fig. 10 shows the results of an electrophoretic run done with histone extracted from regenerating rat liver. It is obvious immediately that the definition in the region of the fast moving α-fraction is even poorer than that in normal rat liver. A very wide band exists in front, that covers the whole area in which the several turquoise bands of

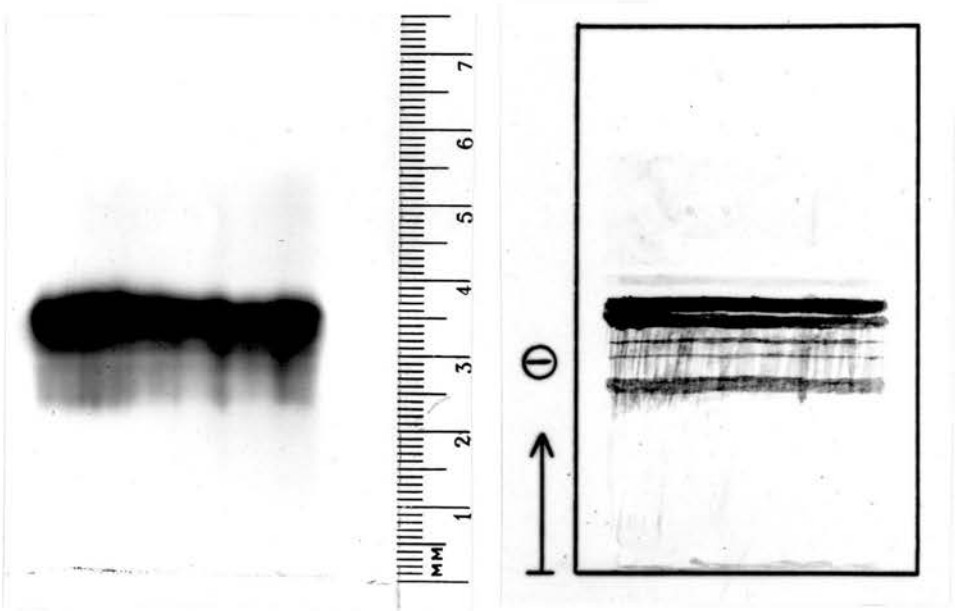


Fig. 10 POLYACRYLAMIDE GEL ELECTROPHORESIS OF REGENERATING RAT LIVER WHOLE HISTONE. LANTHANUM - ACETATE BUFFER PH: 4.9

the α-group are usually seen. The deeply blue-black staining histones of the γ and β-group are as in the case of normal rat liver although the slow moving bands of the β-group are fewer in number. Furthermore material has been left in the origin, presumably aggregated histone that did not penetrate the gel structure. It is difficult to say whether any differences exist between the patterns from normal and regenerating rat liver, although those shown in Figs. 9 and 10 suggest that this might be so. When these two are run adjacently in one gel the only differences observed are in the amount of dye retained by the various fractions that occupy corresponding positions on the gel.

Electrophoresis of rat liver histone fractions

The fractions used in these electrophoretic runs were prepared according to the method of Cruft et al. (1958a). This method was originally used for the fractionation of calf thymus histones, so it had to be modified so that it could be applied to rat liver. The full description of the preparation is given in Chapter Two.

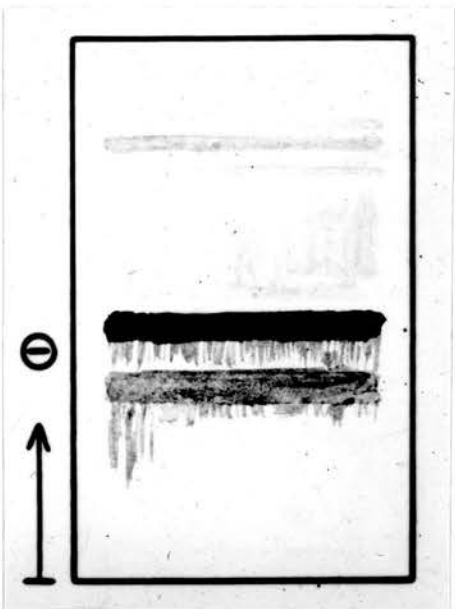


Fig. II POLYACRYLAMIDE GEL ELECTROPHORESIS OF RAT LIVER a-HISTONE. LANTHANUM-ACETATE BUFFER PH: 4·9

α-Histones

In the drawing of Fig. 11 an electrophoretic run for 5 hr. at 4 v/cm. is shown where, as usual a 4% sample of protein has been used. Compared with the equivalent pattern obtained with calf thymus α-histone, this shows fewer bands and poorer definition. The very wide band in the region of the α's is again present and whether it is an entity in itself or is just due to diffusion is difficult to say.

Changes in concentration of α-histone in the sample applied for electrophoresis had very little effect. The same pattern appears either more intensely or more faintly stained following variations of the protein concentration.

β-Histone

The electrophoresis of β-histones from rat liver gave the pattern shown in Fig. 12. A solution of 2% histones in lanthanum-acetate buffer acidified to pH 3 with acetic acid was used, and the duration of the run was 5 hr. at 4 v/cm.

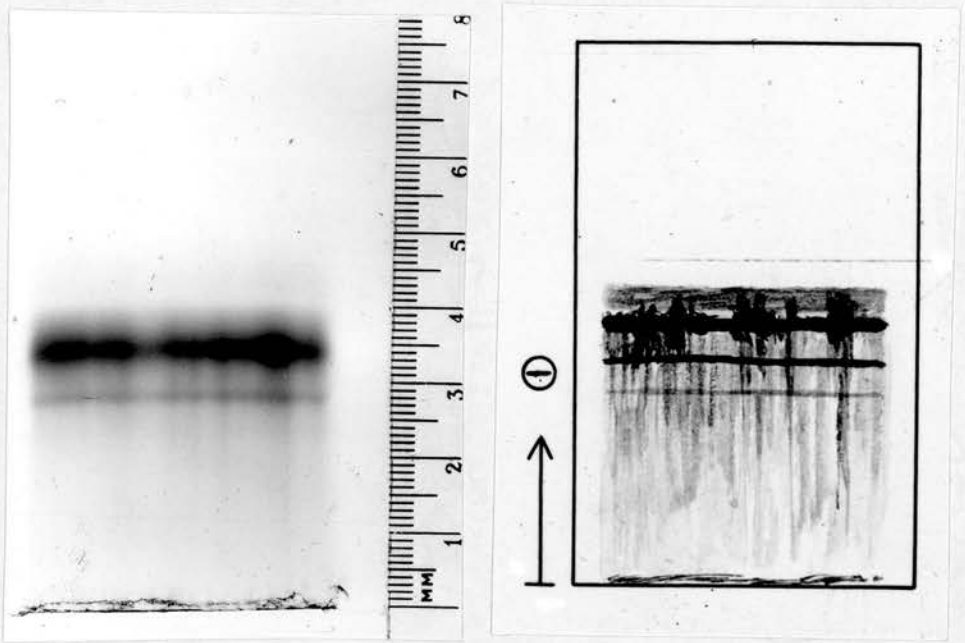


Fig. 12 POLYACRYLAMIDE GEL ELECTROPHORESIS OF RAT LIVER β-HISTONE. LANTHANUM-ACETATE BUFFER PH: 4.9

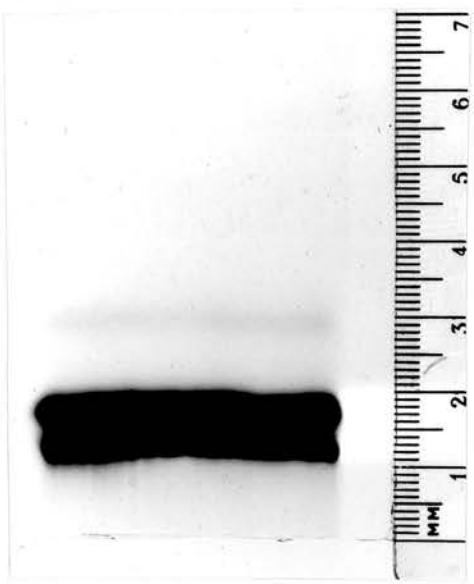


Fig. 13 POLYACRYLAMIDE GEL ELECTROPHORESIS OF RAT LIVER γ-HISTONE. LANTHANUM - ACETATE BUFFER PH: 4.9

Only four bands are visible in this run, there being too much 'trailing effect' and aggregated material in the origin. When a 4% solution of protein is used, the definition is even poorer as the 'trailing' material shadows the edges of the bands. It was found to be essential to leave the sample in solution a minimum of two days before trying an electrophoresis if disaggregation was to be complete. Migrating the proteins for longer periods revealed no more components and served only to decrease the resolution of the bands.

γ-Histone

The γ-histone fraction in polyacrylamide gel electrophoresis gives the pattern shown in Fig. 13 which was obtained with a 4% sample run at 4 v/cm. for 5 hr. The reproduction on the photographic paper is quite accurate and the two typical deeply blue stained γ-bands can be seen. The faint band that is visible ahead of those is due to contamination of the sample by α-histones. There appears to be no trailing material or aggregated material left in the origin.

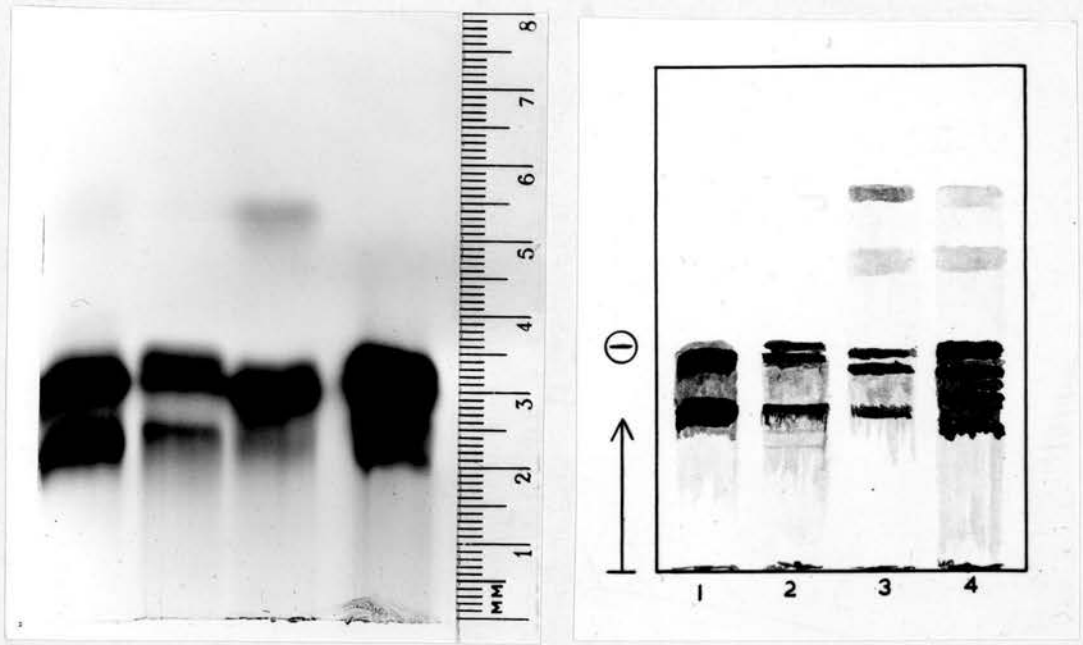


Fig. 14 POLYACRYLAMIDE GEL ELECTROPHORESIS OF RAT LIVER HISTONE FRACTIONS.
LANTHANUM-ACETATE BUFFER PH: 4.9
1 γ -HISTONE
2 β- HISTONE
3 α -HISTONE
4 UNFRACTIONATED HISTONE

Unfractionated rat liver histone run with its three fractions

When the four samples just described individually were run on an polyacrylamide gel the pattern shown in Fig. 14 was obtained. The duration of the electrophoresis was 5 hr. at 4 v/cm. and the samples were 4% in lanthanum acetate buffer, acidified to pH 3, except that of the β-histone which was 2%.

Discussion

Polyacrylamide gel electrophoresis of histones proved to be a very reliable method for identification and comparison purposes. It has constantly yielded results with excellent reproducibility during the whole period it has been routinely applied. Starch gel electrophoresis of histones (Neelin et al. 1959) was a major step towards electrophoretic analysis of this group of proteins, but was unsatisfactory in that the migration distances of the various fractions were found to be exceedingly dependent on sample concentration and moreover that when single bands were eluted and rerun they did not occupy the same position on the gel (Muecke, 1962). The interaction between the free carboxyl groups of the starch gel and the histones is probably responsible for the patterns obtained. In a starch gel containing 0.02 N hydrochloric acid, at the pH of which carboxyl dissociation would be expected to be fully suppressed, whole histone gave only two bands of high mobility (Johns et al. 1961).

These carboxyl groups may also be responsible for the little correlation that exists between the relative migration distances in free electrophoresis and starch gel electrophoresis.

In polyacrylamide gel electrophoresis the α-histones migrate well in front of the rest just as in free electrophoresis, followed by the β and γ-histones. One would expect this to happen in a system as in free electrophoresis where the migration depends on the molecular weight, the shape and charge of the molecules and there are no adsorption effects due to electrostatic interactions between protein molecules and supporting medium. This indeed appears to be the case in polyacrylamide. From all the data available the electrophoresis in these gels is a kind of 'sieving' of the molecules through the gel pores and smaller molecules, other factors being equal, would migrate faster. When the concentration of acrylamide in the gel is lowered (in which case the pore size is increased), the distances migrated are increased but the separation of fractions is poorer.

Hydrogen bonding between the amide groups of the polyacrylamide and carboxyl groups on the protein molecules would seem to be possible and may play a role in the separation of the proteins into bands. When the gels were kept unstained in a methanol-water-acetic acid bath overnight, the bands after staining did not seem to lose any of their sharpness by diffusion and this suggests that they might be held in position by some form of molecular interaction.

The number of zones obtained in electrophoretic patterns is believed to correspond with the number of different kinds of protein molecule in the sample. The possibility that some of the bands, especially the condition sensitive β-group, are aggregates cannot be excluded, although the electrophoretic runs were done under conditions known to suppress aggregation.

It should be added that when histones isolated using widely differing methods were subjected to electrophoresis, the results were the same, and one might expect that, if some of the bands were artifacts of one method, they would not be present in the others.

The comparison of the results obtained by starch gel and polyacrylamide gel electrophoresis is not easy. According to Muecke (1962), who investigated extensively the use of starch gel for the electrophoresis of histones, the 'crude' calf thymus histone gives rise to ten zones. Of these zones one to seven are due to the γ-histones; zones eight and nine are due to the α-histones, and zone ten is due to the β-histone. The material that remained at the origin was explained as aggregated β-histone. When the same histone (isolated in exactly the same way) was run in polyacrylamide gel electrophoresis the pattern obtained showed thirteen zones. In order to identify to which histone group each zone corresponded, the three histone fractions were run individually. From these runs the α-histones gave rise to nine bands (instead of two as in starch gel electrophoresis), the β-histones gave rise to nine also (instead of one), and the γ-histones to three (instead of seven). That is, twenty-one bands in polyacrylamide gel instead of ten in starch gel. The difference is striking as far

as the γ-histones are concerned. With all the variations tried in doing the polyacrylamide gel electrophoresis there was never the slightest suggestion of any more bands appearing in the gel. The explanation must be sought in the structure of starch gel itself, as the samples used for the electrophoreses were identical. It is possible that the γ-histone exists in some strongly aggregated form and, due to the interaction of the starch carboxyl groups, the aggregates split either into smaller ones or into single molecules and so appear in groups of different electrophoretic mobilities. It is equally possible that the observed results are due either to a smaller pore size in starch gel, which allows it to differentiate between the aggregates that the polyacrylamide can't, or to partial denaturation of histone by starch gel changing thus its electrophoretic pattern.

The differences in migration distances between the various bands of the histone fractions are more or less constant. Absolute values cannot be given because, as has already

been mentioned, the gels swell or shrink considerably according to conditions, and although the general pattern does not change, it is impossible to stipulate for example, that the fastest band of the α-group will migrate a specified distance under a certain voltage gradient. Accurate comparisons can only be made by running two or more samples simultaneously on the same gel.

The polyacrylamide gel electrophoresis of rat liver histones, as has already been described, showed poorer results than those obtained with calf thymus histone. No examples of rat liver histone runs in starch gel electrophoresis could be found for comparison in the literature.

The whole histone from rat liver gave rise to less than ten zones (usually eight to nine). The turquoise coloured bands characteristic of the α-group have always been very faint compared with those of calf thymus, and the percentage of α-histone to whole-histone in rat liver has been found to be much less than in calf thymus.

When the fractions of rat liver histone were run individually, α-histone gave rise to a pattern of five bands, β-histone to four bands and γ-histone to two, a total of eleven bands. As far as the γ-histones are concerned the patterns are clear enough to conclude that this is a 'true' result. The α-histones however, although they clearly show five bands, have also a very large lightly staining area. Whether the other zones appearing in calf thymus exist also in rat liver, and for some reason are undetected, is not clear. The aggregated material appears to be more intense than it is in calf thymus histone. In the β-histone electrophoretic patterns the effects of aggregation and trailing very strongly affect the clarity. Further zones may exist but are hidden by the all over staining which occurs between the visible β's and the origin.

PART II

POLYACRYLAMIDE GEL ELECTROPHORESIS IN SYSTEMS RICH IN ORGANIC SOLVENTS

Introduction

Although polyacrylamide gel electrophoresis of histones proved to be an extremely reliable tool for the analysis of histones, further attempts were made to modify it so that it could become suitable for the incorporation of a scintillator into the gel. The basis for these attempts was the finding that histones are soluble in pure ethylene glycol and in glycerol, and also the fact that acrylamide can polymerise in the above solvents.

The solubility of certain proteins in organic solvents or in systems rich in these has been known for a long time. Robertson (1918) demonstrated that the plant proteins zein and gliadin are soluble in 80% ethanol. Zein was found by Swallen and Danehy (1946), to be soluble in twenty-seven non-aqueous solvents, and Rees and Singer (1956) found a further

twelve for zein. The same authors reported that insulin can be dissolved to the extent of at least 1 mg./ml. in some thirteen non-aqueous solvents. 2-Chloroethanol was found to be an excellent solvent for globular proteins by Doty (1959), and was used extensively for conformational studies.

The effects of an organic solvent on a protein molecule seems not to be predictable but to vary from one case to another. Thus ribonuclease was found by ultracentrifugal studies to be extensively aggregated in anhydrous ethylene glycol solutions, containing potassium chloride (Sage and Singer, 1962); while insulin in dilute solution in dimethylformamide or dimethylacetamide is largely dissociated into its true minimal subunits of one A and one B chains (Rees and Singer, 1955, 1956). Insulin was also found to be completely dissociated in 40% dioxan-water (Fredericq, 1957).

According to Singer (1962) the native conformations of proteins seem almost always to be altered to some extent in non-aqueous solvents.

compared with aqueous solutions, and the conformation of any one protein may be different in different solvents.

Sage et al. (1962), who investigated extensively the properties of ribonuclease in pure ethylene glycol containing potassium chloride, reached the conclusion that there are no significant conformational differences detectable on transfer from water to ethylene glycol, apart of course from the aggregation already mentioned.

In good agreement with the above is the work of Manford, Buckley, De and Lively (1962). They pointed out that, with mixtures of ethylene glycol and water, the volume fraction of ethylene glycol required to produce any conformational change in the molecule of β-lactoglobulin was very large.

In view of the findings mentioned above it was considered that it might be reasonably expected that the solution of histones in ethylene glycol and glycerol would not lead to any major conformational changes involving denaturation of the protein.

Experimental

Solutions of histones in ethylene glycol and glycerol were prepared and kept at room temperature. For concentrations of solutions up to 8% (w/v) no difficulty was experienced in the preparation. The chloride salts of histones were, as a rule, more easily soluble than the sulphates and have been used in all cases. In the event of there being a precipitate after a few minutes, the solution was left overnight and then centrifuged. In almost all cases the solution was complete and any insoluble material was mainly impurity (having a yellowish brown colour).

Preparation of the gels

The gels were prepared in exactly the same way as described for the purely aqueous ones in the previous section. The amount of acrylamide was higher, but the ratio of cross-linking agent (N,N'-methylenebisacrylamide) and catalyst (N,N,N',N',-tetramethylethylenediamine), to acrylamide was kept the same.

Gels containing less than 12.5 g. of acrylamide per 100 ml. of solvent were very soft and sticky, and therefore difficult to handle. From 15 to 60 g. of acrylamide per 100 ml. of solvent, the gels have a structure which makes their handling considerably easier than the ones prepared in purely aqueous solvents since they are less brittle and more flexible. In addition to this, cutting a slit with a razor blade gave excellent results every time, the sides having smooth surfaces and hence lessening the chances of small air bubbles being trapped between the paper carrying the sample, and the gel.

The choice of the buffer presented difficulties, because its role would be to carry the current through the gel as well as to keep the conditions in the gel structure unsuitable for histone aggregation. Furthermore the resistance of the gel should ideally be as low as possible to avoid overheating.

The first attempts were made with the lanthanum-acetate buffer pH 4.9 that had already been used. Although the buffer con-

centration was stepped up (ionic strength from 0.02 to 0.1) the migration distance of histones was only a few millimetres in overnight runs.

The next buffer tried was hydrochloric acid-potassium hydrogen pthalate in the pH range 3 to 4 ($\Gamma/2 = 0.07$). The results obtained showed an improvement in the distance migrated (and this could be increased by lowering the pH), but the histone bands, if any, were masked under very heavy trailing effects, and the gels after staining showed a rectangular blue coloured area.

A buffer of lower pH (hydrochloric acid-potassium chloride) was then used. The proportions of acid to salt can be so chosen that a buffer in the pH range of 1 to 2.2 can be obtained. It was noticed that in pH values below 2, the gels became very soft probably because of hydrolysis of the acrylamide. The best results were obtained with a buffer containing 250 ml. of 0.2 M KCl and 33.5 ml. of 0.2 M HCl per litre (pH 2.2 $\Gamma/2 = 0.0567$). The stock buffer solution was prepared in a concentrated form (500 ml. of 0.4 M KCl and

134 ml. of 0.2 M HCl were diluted to one litre) and it will be referred to as 'concentrated HCl:KCl buffer'. It was used diluted four times with water for use in the electrode vessels, and four times with glycerol or ethylene glycol for the preparation of the protein samples.

The solvents consisting of 75% glycerol or ethylene glycol and 25% concentrated HCl:KCl buffer, were found to be the optimum for the electrophoresis, as far as sharpness of band definition, flow of current and heat produced are concerned.

Electrophoretic runs

The electrophoretic set up was identical to the one described in the first section (Fig. 1). All four electrode vessels contained KCl:HCl buffer pH 2.2 and the current applied was the maximum that the gels could tolerate without overheating, viz. 8 v/cm. The samples, which were prepared in the same solvent as the gels, usually contained 4% (w/v) protein and the electrophoretic runs lasted for periods of 18 - 20 hr. using no cooling.

Staining, destaining and dehydration of the gels

The gels were stained with a 0.5% (w/v) solution of Amido Black 10B in water-methanol-acetic acid 5:5:1, for a period of at least 40 min. and were always destained electrophoretically. This increase in the staining time was found to be essential because of the very low speed at which the dye diffuses into the gel. The electrophoretic destaining was also found to be necessary because otherwise the gels needed three to four days to clear. This was done by the method detailed in the first section (Fig. 2). The dehydration of the gels was also carried out as described, (Fig. 3), and the presence of the organic solvents made no difference at all to the results.

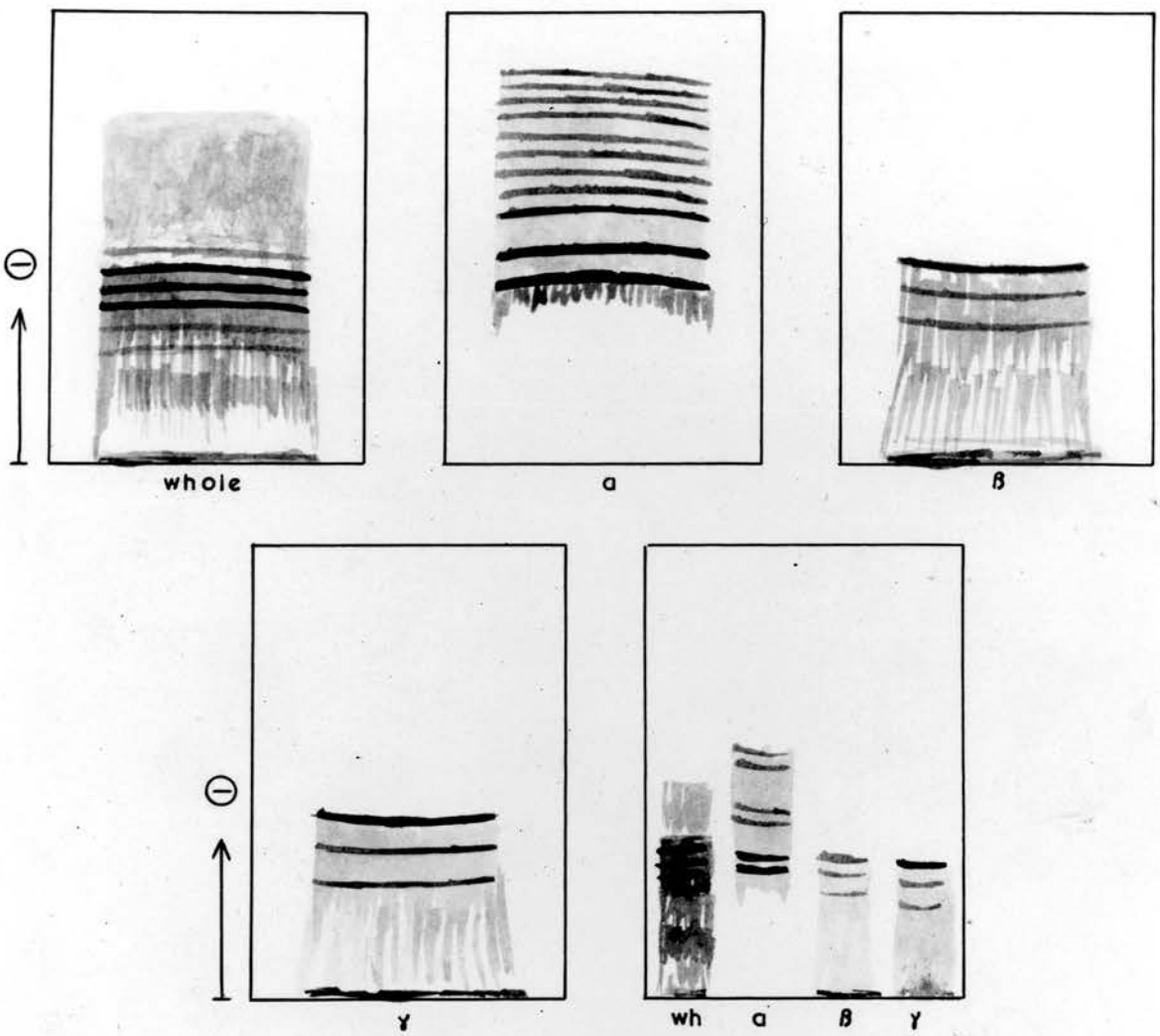


Fig. 15 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS HISTONES. GELS AND HISTONE SAMPLES PREPARED IN GLYCEROL 75%, AND THE CONCENTRATED HCl:KCl BUFFER PH:2.2, 25%. RUN FOR 18 HOURS AT 8 V/cm.

Results

Gels containing glycerol

The gels were prepared as already described and the protein samples used have in all cases been 4% (w/v). The electrophoretic runs were done under identical conditions for 18 hr. at 8 v/cm.

The whole (unfractionated) calf thymus histone after staining showed six histone bands (Fig. 15). From the relative positions of the bands, it was noticed that the heavy staining bands were clearly visible, whereas the areas occupied by the less intense α's and β's were heavily shadowed by trailing material and no zones could be detected. A lot of aggregated material was left at the origin.

The situation became clearer when histone fractions were used. The α-histone fraction revealed eleven bands distinctly separated from each other. This number compares favourably with the nine bands obtained in purely aqueous systems. The spaces between the bands are quite heavily shadowed and for this reason photographs became impracticable. No

aggregated material was left at the origin and the trailing effect behind the bands was not at all heavy. In some cases the curving of the bands was more pronounced than in the run shown in Fig. 15 but the patterns were unchanged. Variations in the concentration of α-histone in the sample produced the same number of zones, though of different intensity. At a concentration below 3% (w/v) the faster bands were almost invisible and the shadowing of the spaces between them, although less intense, were still there. At a concentration of over 6% the only zones distinguishable were the faster three; the rest showed no definition because of heavy smear.

The electrophoresis of β-histones, done in the same way, resulted in the pattern shown also in Fig. 15. The number of bands was only three, compared with the nine obtained in purely aqueous systems, and the trailing material in the whole area swept by the migrating proteins was deeply stained so that any bands present in this area would not be distinguishable. Apart from the fastest band

of the β-group, that stains deeply blue with Amido Black, the rest stain very faintly, and minor shadowing masks them. The amount of aggregated material left in the origin is enormous compared to that seen in purely aqueous systems.

The electrophoresis of γ-histones, under the same conditions, resulted in the separation of this fraction into three bands (Fig. 15). The patterns of the β and γ-histones are similar, having the same number of bands in almost identical positions. But the bands of the γ-fraction are more heavily stained and are always more clearly distinguishable despite heavy trailing and shadowing effects. Considering the fact that γ-histone in purely aqueous systems yields two bands, of which one is double, this system containing glycerol is thought to be an improvement. Whether the third band clearly separated is a sub-fraction of the γ-fraction, or an artifact of the method is difficult to say. But the mild conditions used throughout, suggest that it is a 'true' protein fraction.

In Fig. 15 an electrophoretic run of whole calf thymus histone and its fractions run in neighbouring positions in the one gel, is included, when the relative positions of the various bands can so be seen.

α-Histone was the fraction chosen to be used in the further investigation of the electrophoresis of histones in systems rich in glycerol. The reason for this choice was that this fraction shows more bands than the other two and the pattern is better defined with much less trailing effect and uncertainty.

Effect of glycerol concentration on the pattern of α-histones

Several gels were prepared in glycerol and KCl:HCl buffer containing 10 - 60% (v/v) glycerol. In all the gels the acrylamide concentration has been 15 g. per 100 ml. of solvent. The samples were prepared by dissolving the α-histone in the same solvent as the respective gel, and 4% (w/v) α-histone samples were used throughout.

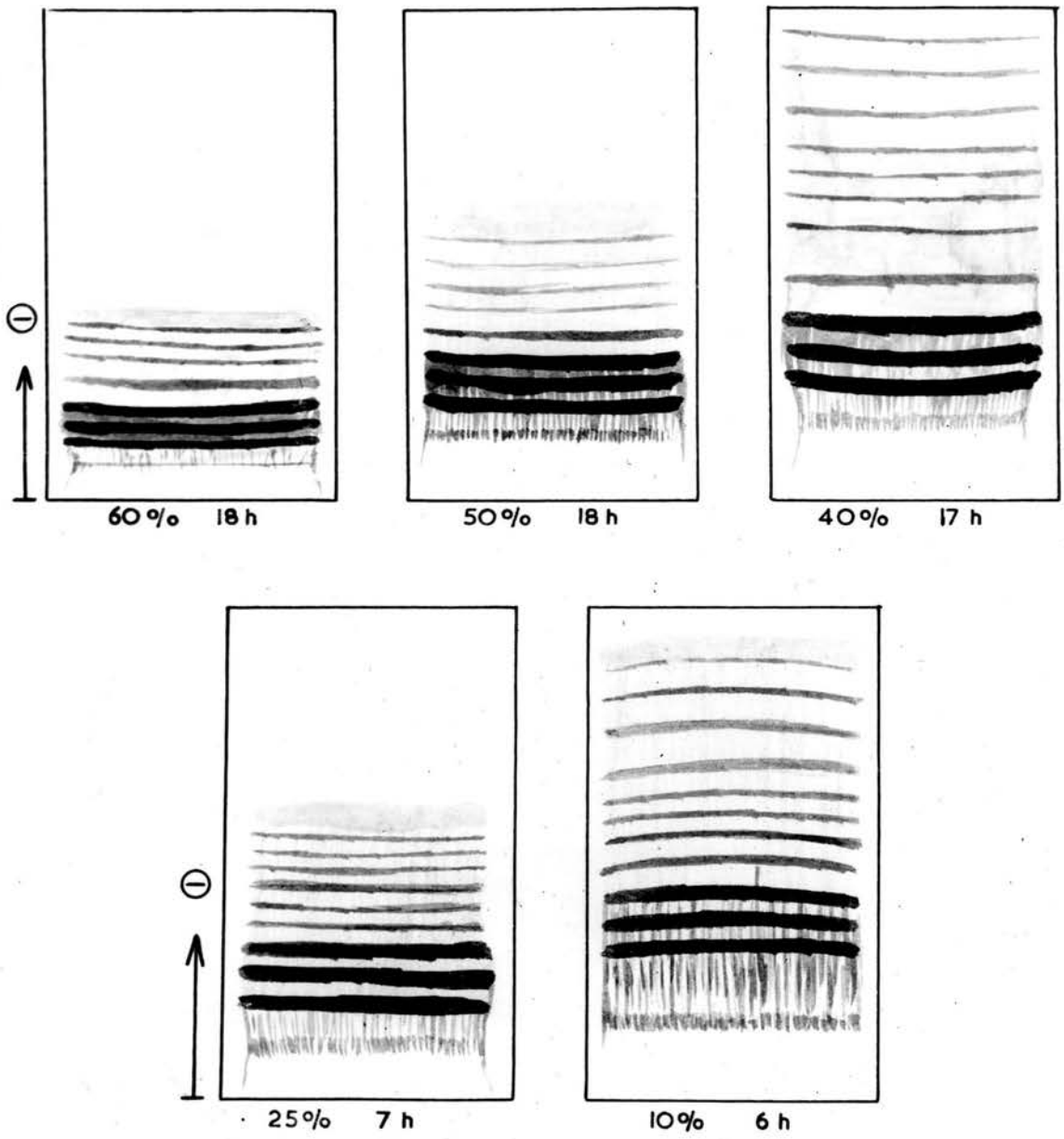


Fig. 16 EFFECT OF GLYCEROL CONCENTRATION ON THE ELECTROPHORETIC PATTERN OF a- HISTONE.
THE FIGURES UNDER EACH DRAWING REPRESENT THE PERCENTAGE (v/v) OF GLYCEROL IN THE SOLVENT, AND THE DURATION OF THE RUN IN HOURS.

Fig. 16 displays the results of five electrophoretic runs. The concentration of glycerol used in each case is shown together with the length of time, in hours, of the electrophoretic run. It was found necessary to cut down the duration of the electrophoresis as the glycerol concentration decreased, because the proteins would otherwise migrate out of the gel. More extended runs did not at all improve the resolution of the bands.

It was noticed immediately that the relative migration distances of the various bands was altered. From the drawing shown in Fig. 15 the distance covered by the fastest α-band is roughly twice that of the slowest. By reducing the glycerol concentration to 60% (Fig. 16) the distance migrated is increased three-fold. The resolution of the bands in the 60% glycerol gels was good although the bands were only seven in number compared to eleven in 75% glycerol gels. The sharpness of the bands is excellent though again the spaces between the bands are rather heavily stained.

The situation with the 50% glycerol gel is similar. One more band (eight in all) appears in front of the rest. The trailing material throughout the gel still exists and the spaces between the bands are shadowed.

Further reduction of the glycerol concentration to 40% produced the pattern displayed in Fig. 16. The difference between this one and the previous two is striking. The three heavily stained slow bands have not migrated much further than in the two previous runs, but the rest of the fractions have moved much faster and the area they occupy has extended. The bands now numbered eleven, all very sharp, but again the smear effect was very noticeable. It was also noticed that the shadowed part, which migrates ahead of the bands in the two previous runs, has disappeared, and probably gave rise to the three extra bands.

The situation was not improved in a 25% glycerol gel. The bands were reduced to nine, the pattern approached that given by purely aqueous systems, and the shadowed area had again appeared ahead of the bands.

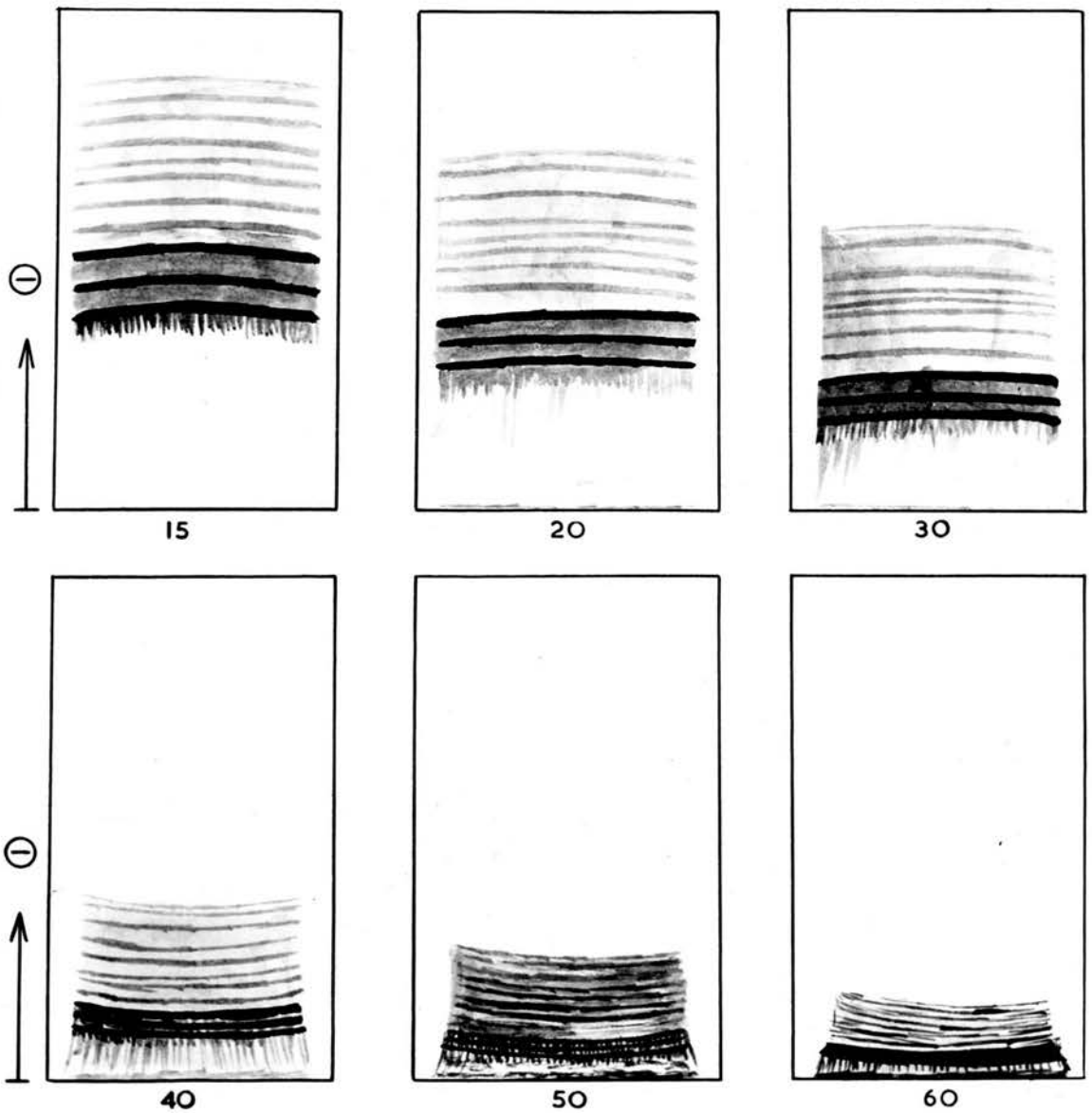


Fig. 17 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS a-HISTONE. GELS PREPARED IN 75% GLYCEROL, AND 25% CONCENTRATED KCl:HCl BUFFER. THE CONCENTRATION OF ACRYLAMIDE IN GRAMS PER 100 ml OF SOLVENT IS GIVEN UNDER EACH DRAWING.

Further reduction of the glycerol content to 10% resulted in the pattern included also in Fig. 16. This, again, is close to the patterns obtained in purely aqueous systems; the number of the bands is eleven and the smear ahead of the bands is present. For the first time the trailing material left behind is quite intense.

Effect of acrylamide concentration on the pattern of α-histones

The experiments were done in 75% glycerol as the results of these gels had been found to be the most satisfactory of all, giving very clear electrophoretic patterns and the highest number of bands, together with the least trailing material and smear ahead of the bands.

The results of six runs, done in gels with different acrylamide concentrations are shown in Fig. 17. The amount of acrylamide in grams per 100 ml. of solvent is shown under each drawing. In all the cases 4% (w/v) α-histone dissolved in 75% glycerol and 25% concentrated HCl:KCl buffer, was used. The voltage gradient was 8 v/cm. in all cases and the duration of all the runs 18 hr.

The effect of the increased acrylamide content in the gel is immediately felt as the gels become harder and lose some of their elasticity. The gel containing 60 g. of acrylamide per 100 ml. of solvent is hard and brittle but still possible to handle although the slit cut with a razor blade is not always smooth surfaced.

The effects on the patterns given by the α-histones are what would be expected. The distances migrated became shorter, and the bands sharper. The shadowing of the spaces between the bands became much more intense, and in the last two cases (50 and 60 g. of acrylamide respectively) it was very difficult to identify the bands. The trailing effects also increased on increasing the acrylamide concentration and the amount of material left at the origin, obviously not being able to penetrate the gel, increased too.

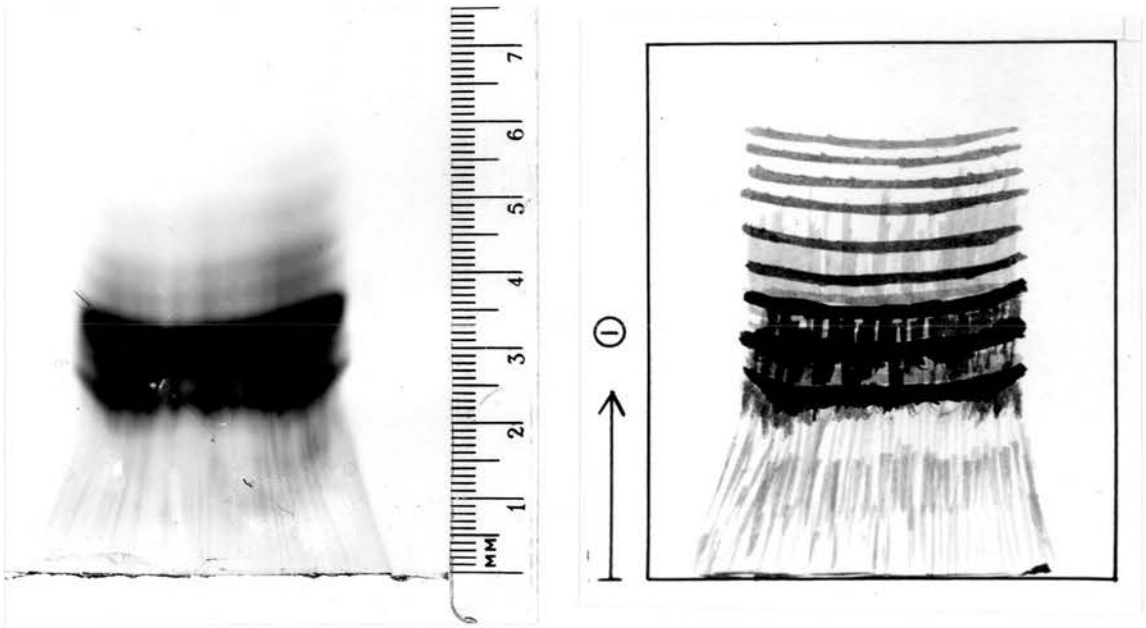


Fig. 18 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS UNFRACTIONATED HISTONE.
ETHYLENE GLYCOL GEL, HCI:KCI BUFFER PH: 2.2.

Gels containing ethylene glycol

The experiments with gels containing ethylene glycol were performed in exactly the same way as those with glycerol.

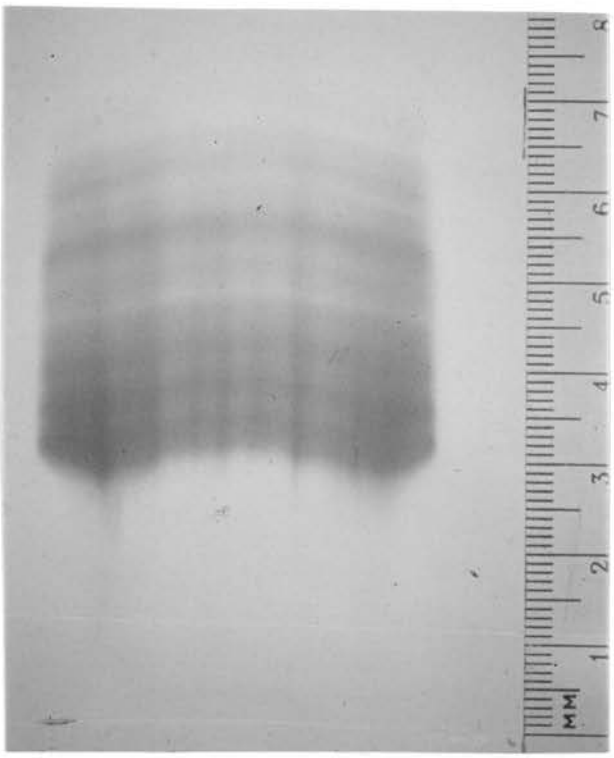
The gels and the samples were prepared using the same KCl:HCl buffer pH 2.2 either in ordinary or the concentrated forms. The chloride salts of the histones were used throughout and the whole histone or its fractions were the same as used for the experiments with glycerol. The gels containing ethylene glycol were more easily prepared (due to the difference in viscosity between the two solvents), and softer. They were also less brittle and in general showed improved handling qualities.

Unfractionated histone

The unfractionated histone, on an electrophoretic run in a gel containing 75% ethylene glycol and 25% concentrated KCl:HCl buffer, produced the pattern shown in Fig. 18. The protein concentration in the sample was 4% (w/v) in the same solvent and the run lasted

18 hr. at 8 v/cm. Again these were found to be the optimal conditions for the same reasons as described for the glycerol containing gels.

In the pattern, nine bands in all can be seen; of those the six faster belong to the α-histone having the typical turquoise colour of this histone group, and the remaining three are the two main γ-fraction bands and one β. Between the slowest bands and the origin the trailing effects are very strong, and no bands can be seen in the region which is usually occupied by the slow β-fraction. The difference between this run and its equivalent in a gel rich in glycerol (shown in Fig. 15) is the better definition and the greater number of bands (nine instead of six.) The α-bands ahead of the rest are very clearly defined and the shadowing of the spaces between the bands is much less intense. However the curving effects are more pronounced in ethylene glycol, although this is certainly not serious enough to affect the resolution.



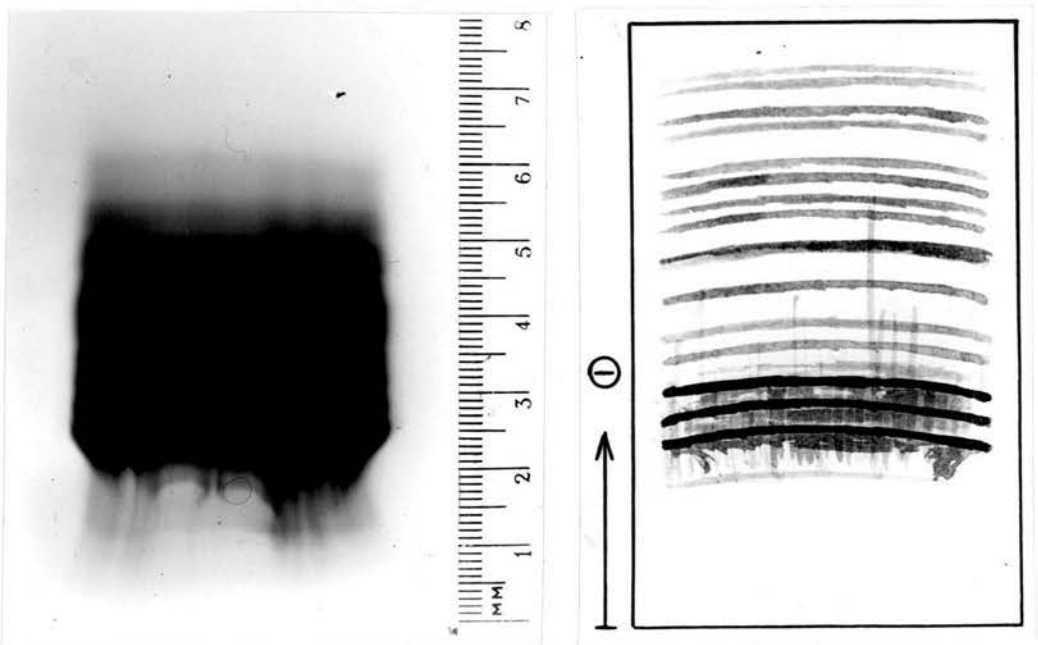


Fig. 19 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS a-HISTONE.
ETHYLENE GLYCOL GEL, HCl:KCl BUFFER PH: 2·2 .

α-Histone

When α-histone was run the results obtained are those shown in Fig. 19. Two photographs, both of the same gel are included, one in colour, and also a sketch of the pattern as it actually looked. The reproduction of the photographs is very deceiving and these are included only in order to give a rough idea of what the gels look like. The trailing effects are completely absent from the coloured print, while the resolution is not discernable in the other one. The very fast α-bands are not visible in either and their position is only shown in the drawing. The spaces between the bands are not stained to the extent suggested by the colour print and the bands are well defined and sharp. The number of bands, compared to the purely aqueous gels, is increased. In the region of the gel occupied by the α-fraction fifteen bands are clearly seen, although two more are present in a position which is normally occupied by the bands of the β-group. These two bands do not seem to be impurity because when the same α-fraction is

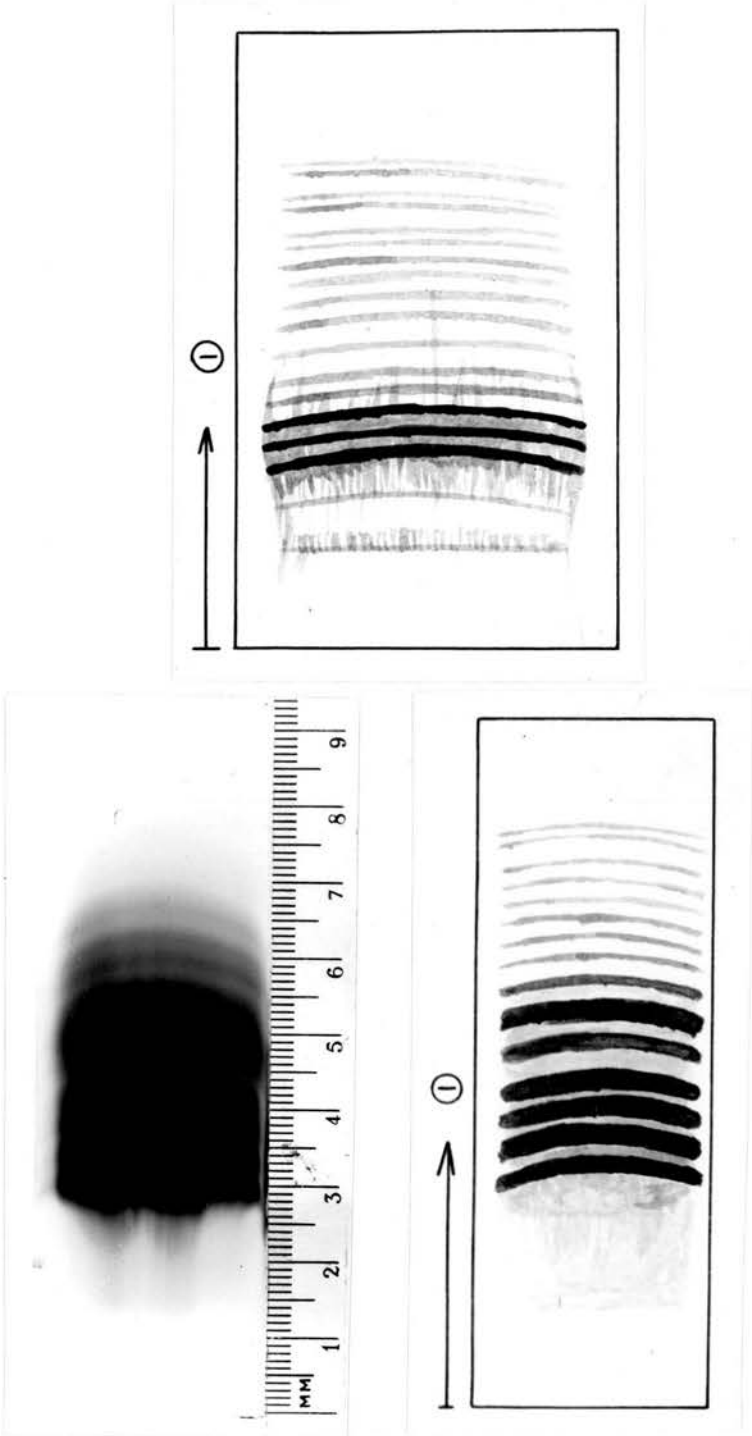


Fig. 20 CURVING OF THE BANDS OF THE CALF THYMUS α-HISTONE PATTERN IN ETHYLENE GLYCOL GEL. HCl : KCl BUFFER PH: 2·2 .

run in a purely aqueous system the pattern obtained is the one shown in Fig. 5. Furthermore the two slow bands in question are not always present as two distinct entities. The faster one is always visible in the same position, but the slower one sometimes only appears as a zone of more intense staining than the neighbouring areas, and lacks sharp definition. The amount of material left in the origin is very little and occasionally nil. The trailing effects are slight and are restricted to the area between the three heavier staining bands and the origin; sometimes they stop at the slowest band.

The curving effects, most probably due to unequal heating of the gel, are not steady and are more pronounced in some cases than in others. Two typical cases are shown in Fig. 20. This phenomenon never occurs to any great extent in the case of α-histones, and in some gels the direction of curvature is reversed so that the edges of the bands migrate further than the middle part. This occurs more often with the

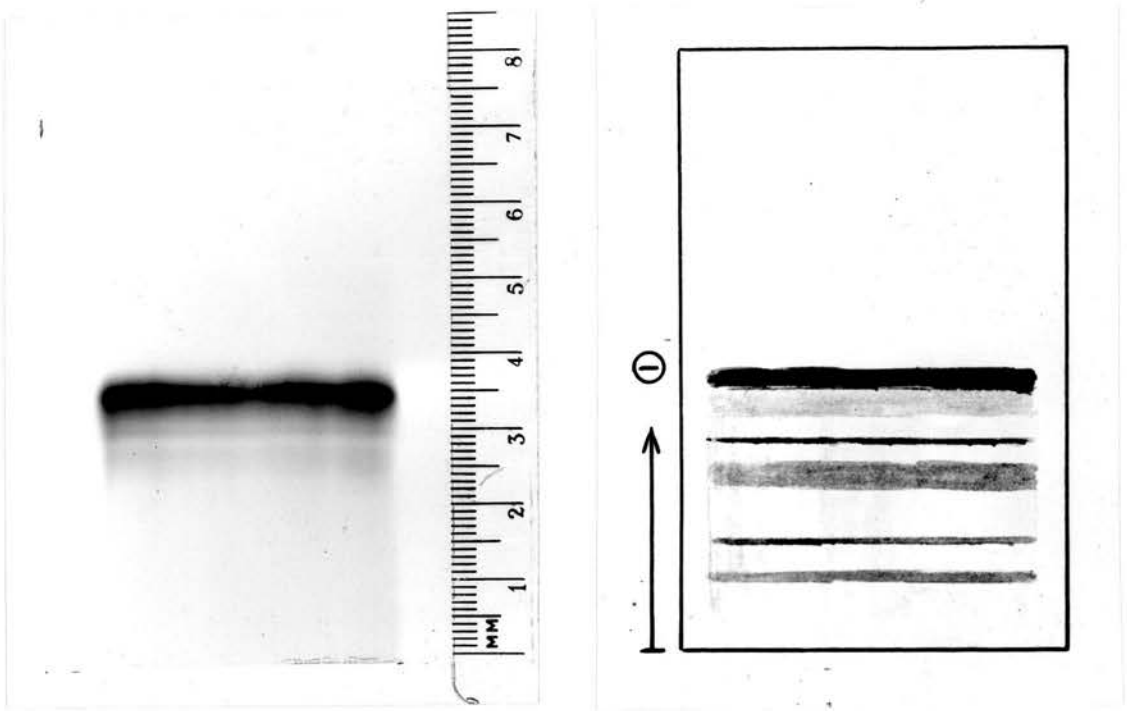


Fig. 21 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS β-HISTONE.
ETHYLENE GLYCOL GEL, HCl:KCl BUFFER PH: 2·2 .

other histone fractions. Variation of the amount of protein in the sample applied for electrophoresis did not improve the pattern. When the protein was more than 4% (w/v) the bands were more heavily stained, as were the spaces between them, and the trailing effects were intensified. If the sample used contained less than 3% (w/v) protein, then most of the fast-moving α-bands disappeared.

β-Histone

The electrophoresis of β-histone carried out under conditions identical to those used with α-histone, produced the pattern shown in Fig. 21. Again the differences between the actual pattern on the gel and the photographic reproduction are enormous. The drawing is included in order to show the details of the pattern.

Six bands are clearly distinguishable in this run, all having sharp edges and showing good definition. There are noticeable differences in the staining intensities of the various bands and the two slower bands are just visible against the shadowed space left behind

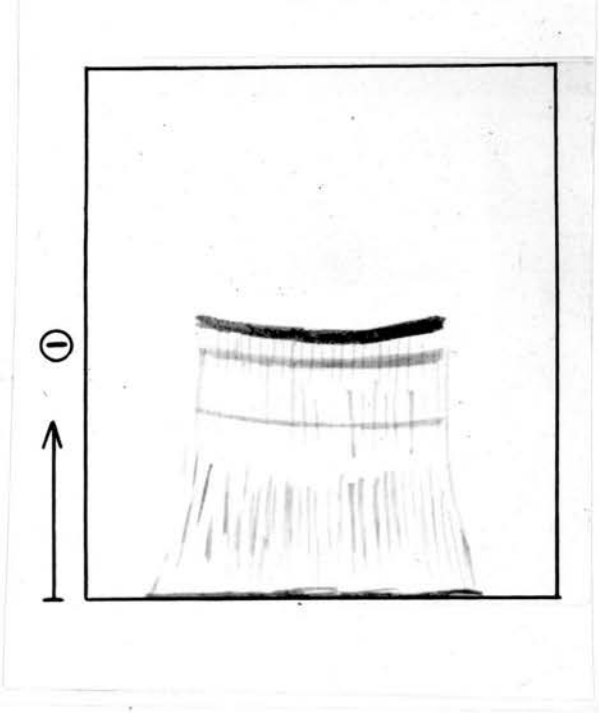


Fig. 22 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS γ-HISTONE.
ETHYLENE GLYCOL GEL, HCl:KCl BUFFER PH:2.2 .

the proteins throughout the area of migration. There does not seem to be a particularly large amount of material left in the origin. The run was done with a 4% protein sample, and variation of the protein content had the expected results, although with more effect than usual. On increasing sample protein concentrations from 5 to 8% (w/v) the patterns became progressively darker and the bands less well defined, until at a concentration of 8% the resolution disappeared completely. At concentration of 2% only two bands were clearly visible, the others being very faint and not easily distinguished.

An increase (or decrease) in the duration of the run did not make much difference to the pattern.

γ-Histone

The electrophoresis of γ-histone produced a pattern of three bands (Fig. 22). This pattern is similar to the one given by gels rich in glycerol and quite different from the ones obtained in aqueous gels. The three bands

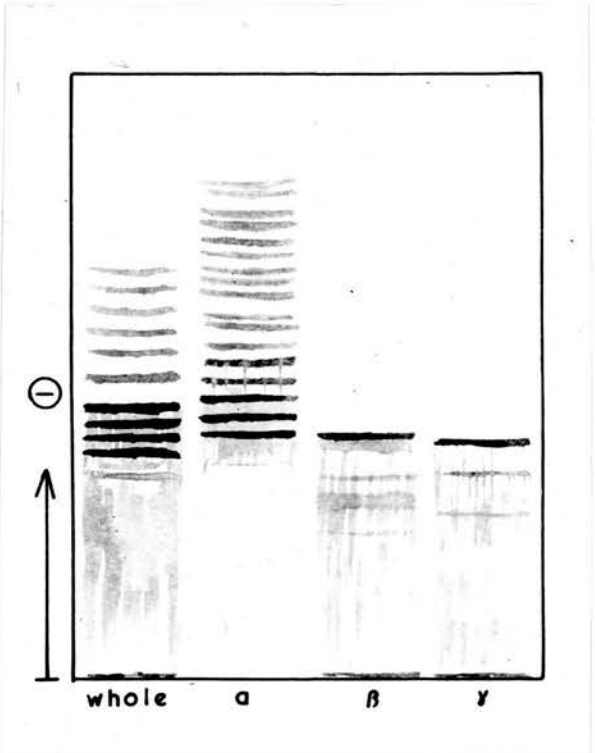


Fig. 23 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS WHOLE HISTONE, AND HISTONE FRACTIONS.
ETHYLENE GLYCOL GEL, HCl : KCl BUFFER PH: 2·2.

are distinctly separated, and very much less intensely stained than usual. For this reason a photograph of this gel gave no results. The trailing effects are quite intense and material left at the origin is also clearly visible. The sample used contained 4% (w/v) γ-histone and was run for 18 hr at 8 v/cm.

Improvement of the pattern has not been possible by varying the sample protein concentration or the duration of the electrophoretic run.

Unfractionated histone adjacent to its fractions

The drawing of the pattern obtained from this run is shown in Fig. 23 when the samples used were 4% (w/v) protein solutions, run for 18 hr. at 8 v/cm.

Effect of ethylene glycol concentration on the electrophoretic pattern of α-histones

In order to investigate the effect of varying the ethylene glycol concentration, α-histone was the fraction chosen for the reasons outlined in the corresponding section on glycerol.

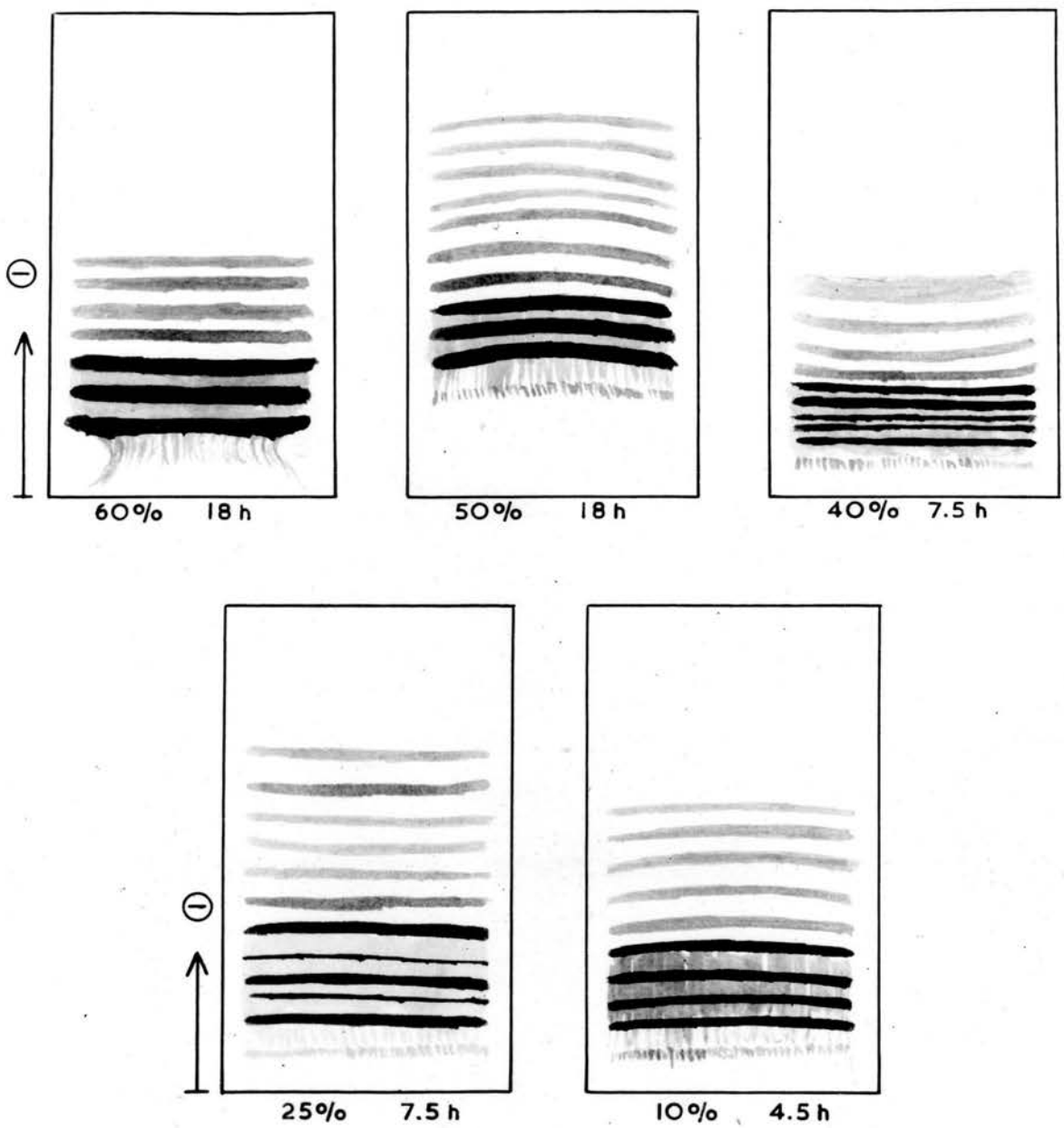


Fig. 24 EFFECT OF ETHYLENE GLYCOL CONCENTRATION ON THE ELECTROPHORETIC PATTERN OF CALF THYMUS α-HISTONES. THE FIGURES UNDER EACH DRAWING REPRESENT THE PERCENTAGE (v/v) OF ETHYLENE GLYCOL IN THE SOLVENT, AND THE DURATION OF THE ELECTROPHORETIC RUN IN HOURS.

The gels were prepared in aqueous solvents containing from 10 - 60% (v/v) ethylene glycol and 25% (v/v) concentrated KCl:HCl buffer pH 2.2. Solutions of calf thymus α-histone were prepared in the same solvent as the gel and contained 4% (w/v) protein. The patterns obtained are shown in the drawings of Fig. 24. Under each pattern the amount of ethylene glycol, per cent, is shown together with the time in hours each electrophoresis lasted. As the distances migrated were restricted by the length of the gel the time had to be shortened for the more dilute ethylene glycol gels. The voltage gradient in all cases was kept constant at 8 v/cm.

The immediate result of the reduction of the ethylene glycol content of the solutions was a reduction in the number of bands of increased width. The electrophoresis in systems containing 75% ethylene glycol produced patterns showing fifteen to seventeen bands (Fig. 19). The reduction of ethylene glycol to the level of 60% reduced the number of bands to seven, the same sort of striking difference as has been noticed in the case of glycerol. The

distance migrated was, as in the case of glycerol, found to be shorter, and the intensity of the band colourations was greater.

When the ethylene glycol content was further lowered to 50%, the migration distance increased and so did the number of bands; in this case ten or eleven were distinguishable. The bands were not as wide as with 60%, and their resolution again was excellent with less trailing effects.

Further reduction of the ethylene glycol to 40%, produced a pattern showing nine bands. The fastest band was the widest of all and the reduction in the number of bands might be explained as the 'joining' of two or more bands. This is supported by the fact that some of the bands are now wider than with 60% and that in the region of the slow, heavily staining ones, two new bands have appeared. The resolution is not as good as in the previous runs with more ethylene glycol, and the colour contrast of the bands, is now greater. No material was left in the origin.

The electrophoresis done in gels containing 25% ethylene glycol gave the pattern shown in Fig. 24. The bands occupy a still longer area and are now more numerous (eleven). The resolution is improved and the shadowing and trailing effects less intense than in the case of 40%.

When a gel containing 10% ethylene glycol was used (Fig. 24), ten bands were well distinguished and the trailing effects limited to the slower bands. The pattern is more or less like the one obtained by using gels prepared in purely aqueous systems, although one more band is visible in the ethylene glycol gel.

Effect of the acrylamide concentration on the electrophoretic pattern of α-histones

The gels were prepared in 75% ethylene glycol and 25% concentrated KCl:HCl buffer pH 2.2. The amount of acrylamide used was from 15 - 60 g. per 100 ml. of solvent. In all the cáses a 4% (w/v) solution of calf thymus α-histone prepared in the same solvent was used, and the electrophoretic runs were

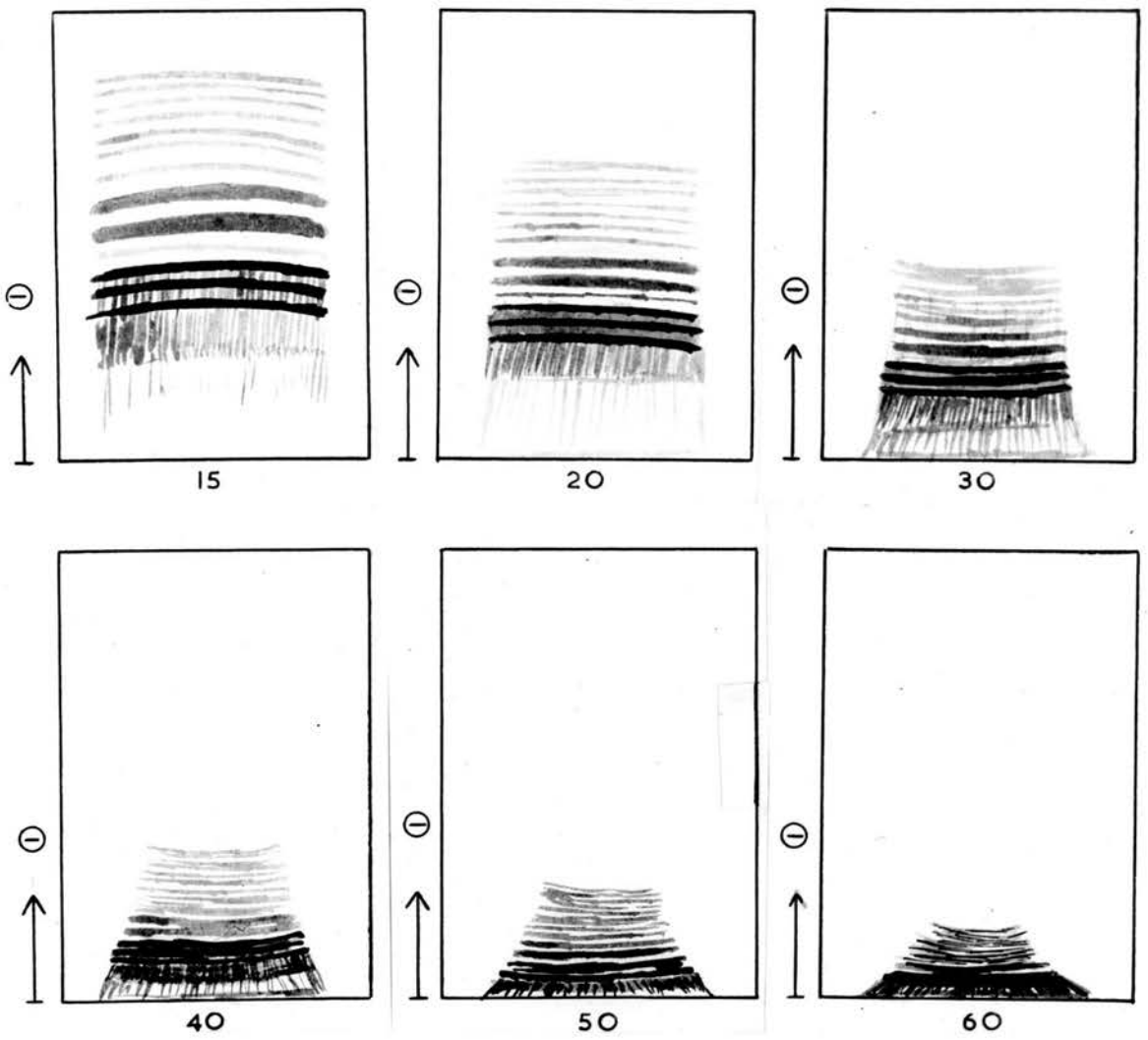


Fig. 25 POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALF THYMUS α-HISTONE. GELS PREPARED IN 75% ETHYLENE GLYCOL, 25% CONCENTRATED KCl:HCl BUFFER.
THE CONCENTRATION OF ACRYLAMIDE IN GRAMS PER 100 ML OF SOLVENT IS GIVEN UNDER EACH DRAWING

done for 18 hr. at 8 v/cm. The patterns obtained are shown in the drawings of Fig. 25. The 15 g. gel is included for the sake of comparison, although it has already been discussed in detail.

From the results obtained when a 20 g. gel was used (Fig. 25) it is immediately noticed that the trailing effects are heavier and some material is left behind at the origin. The distance of migration is now shorter, the bands are narrower and sharper in resolution but are also fewer. Thirteen only could be counted instead of fifteen.

By increasing the acrylamide content to 30 g. per 100 ml. of solvent the picture becomes more obscure. The migration is further shortened, the staining of all the components far more intense and their definition worse. The bands are no longer clearly distinguishable, the shadowing and trailing effects are very pronounced, and more material than before is left at the origin. The curvature of the bands is also increased and the number has more probably decreased. Due to poor definition they could not be counted with certainty.

Further increase in the amount of acrylamide to 40, 50 and 60 g. per 100 ml. of solvent made the gels harder and more brittle. The migration distances were further decreased and the resolution of the bands almost non-existent. The curving effects became much more intense, the smear covering the whole area very heavy and there was a lot of material that did not penetrate the gel, remaining at the origin.

When the amount of acrylamide used was more than 60 g. the gels became extremely brittle and impossible to handle.

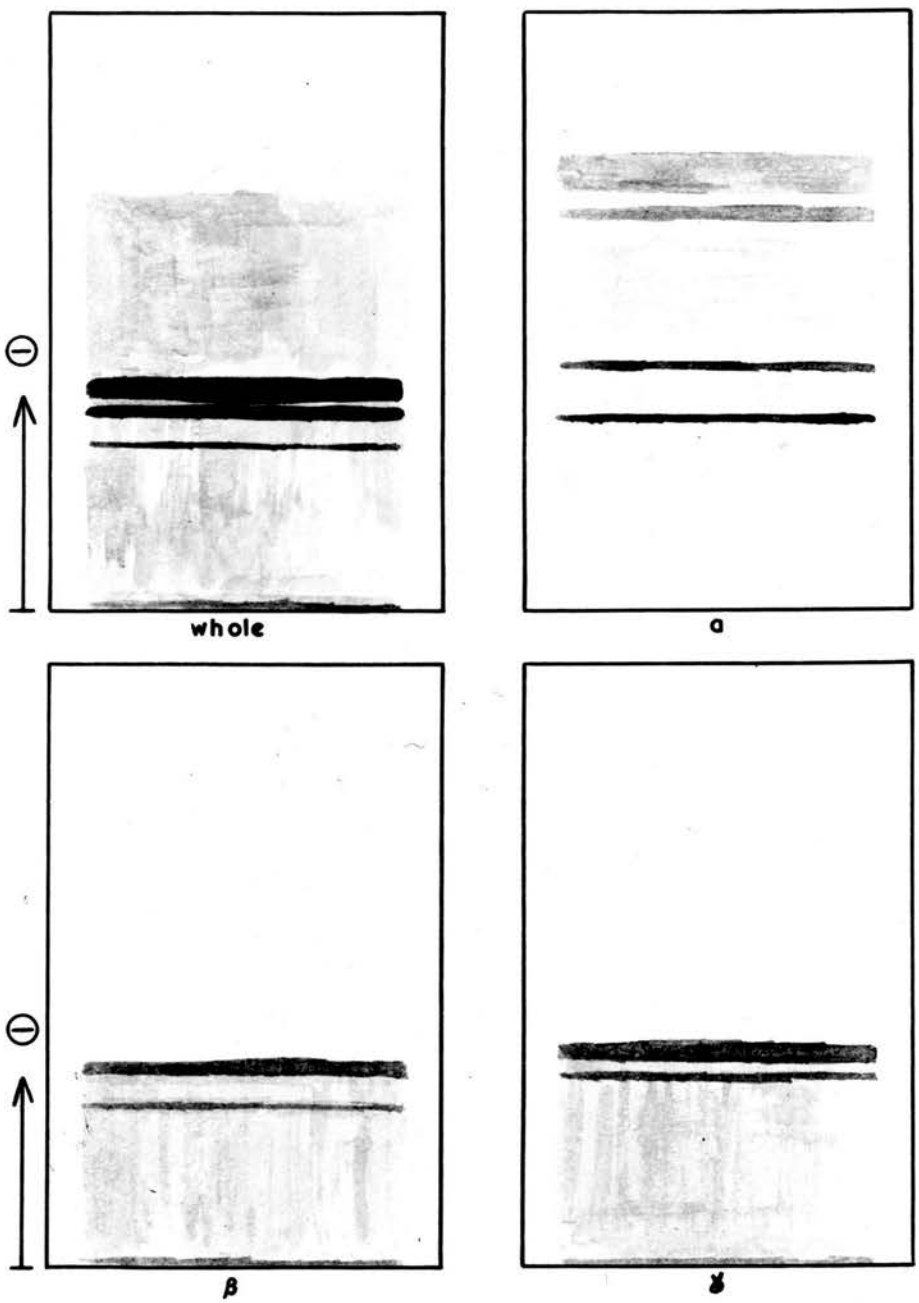


Fig. 26 ELECTROPHORESIS OF RAT LIVER HISTONES. POLYACRYLAMIDE GELS PREPARED IN 75% GLYCEROL WITH 15 GR. ACRYLAMIDE PER 100 ML OF SOLVENT. RUN FOR 19 HOURS AT 8 V/cm.

ELECTROPHORESIS OF RAT LIVER HISTONES

Following the results obtained with calf thymus histones in systems rich in organic solvents, rat liver histones were tried under similar conditions. In all cases the gels were prepared in 75% organic solvent and 25% concentrated KCl:HCl buffer. The proteins were dissolved in the same mixture. It was observed that the histones extracted from rat liver nuclei were not as easily dissolved as the ones extracted from calf thymus. The histone fractions were obtained by the same technique as before which is described in detail in the next section.

Gels with glycerol

Unfractionated histone

The unfractionated histone from rat liver, when run, gave the pattern shown in Fig. 26. Only three bands can be seen. The fast moving α-fraction gave rise to no bands at all. Instead the whole area was shadowed with the dye retained by diffused material. This run was performed with a 4% (w/v) sample, for 19 hr.

at 8 v/cm. Varying the conditions, by increasing or reducing the sample protein content and changing the duration of the electrophoresis resulted in no improvement at all. Neither were the results improved when gels of different glycerol or acrylamide concentrations were used.

α-Histone

The α-histone, when run under identical conditions, showed a pattern of four bands. The two more heavily stained bands are clearly visible, but not the two fast ones (Fig. 26). The whole gel area has a smear, and there is no material left behind at the origin. Attempts to improve the results and obtain clearer patterns failed. The variations in the conditions used were those described above for rat liver whole histone electrophoresis. The run shown was done with a 4% (w/v) sample at 8 v/cm. for 19 hr.

β-Histone

A drawing of the electrophoretic run obtained with β-histones is included in Fig. 26.

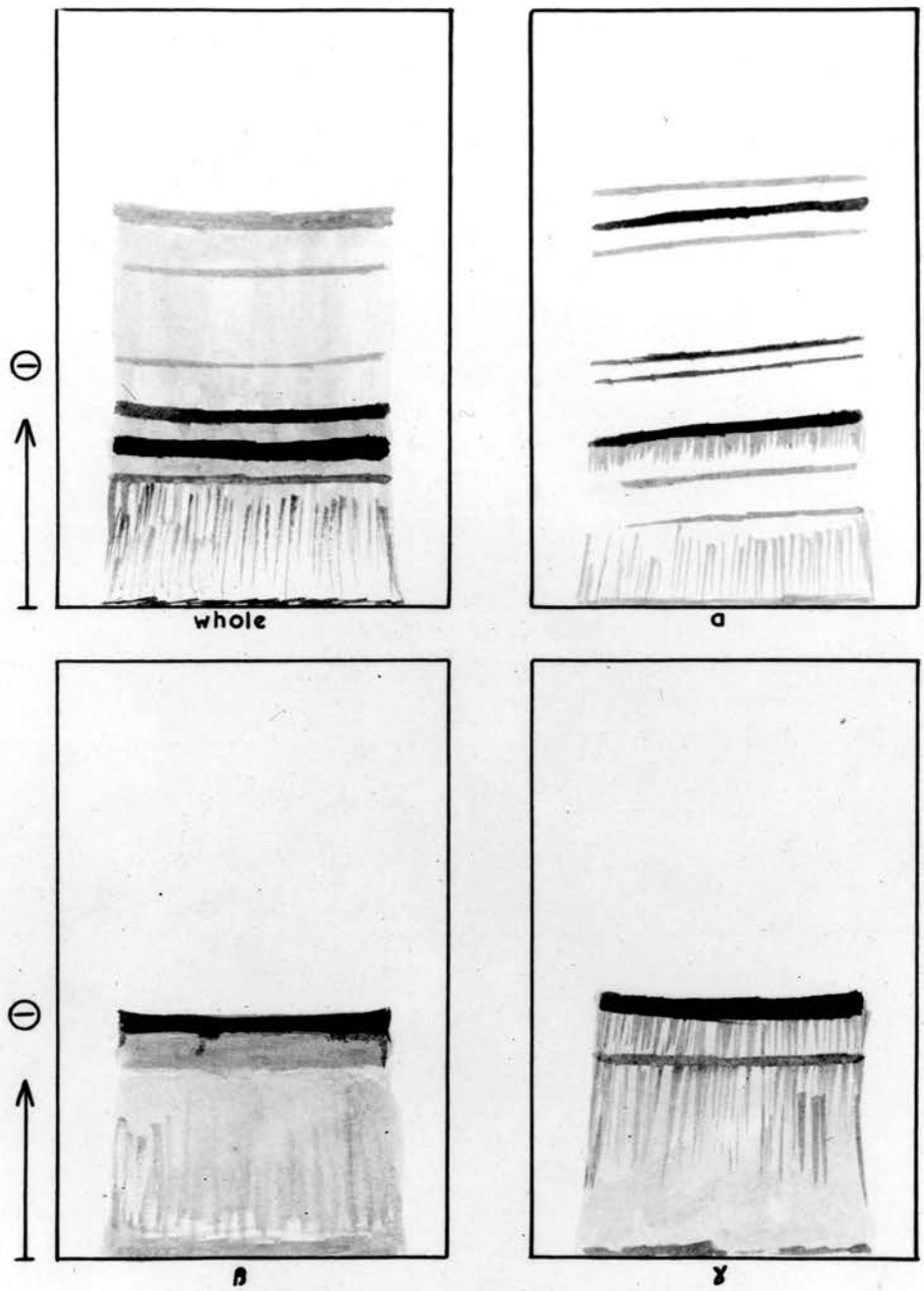


Fig. 27 ELECTROPHORESIS OF RAT LIVER HISTONES. POLYACRYLAMIDE GELS PREPARED IN 75% ETHYLENE GLYCOL WITH 15 GR. ACRYLAMIDE PER 100 ML OF SOLVENT. RUN FOR 19 HOURS AT 8 V/cm.

This run was done with a 4% β-histone sample for 19 hr. at a voltage gradient of 8 v/cm. Two bands only are visible. There is a lot of aggregated material at the origin and the space between the origin and the bands shadowed. Improvements on this pattern were not achieved under any of the circumstances tried.

γ-Histone

This fraction gave the patterns also shown in Fig. 26. It consists of two bands and is similar to the one of the β-histones. The bands however are more heavily stained and are closer together. The shadowing effect is more intense, and material left at the origin is also present. Again all the attempts to improve on this pattern failed.

Gels with ethylene glycol

Unfractionated histone

The electrophoresis of rat liver whole histone gave the pattern shown in Fig. 27. Six bands in all are clearly distinguishable. In the region where the bands of the α-fraction are expected to migrate, three bands exist, and

the rest of this area is lightly stained. The intensely staining α-band and γ-band are both well defined as is a slower band of lighter staining, most probably the slow γ-band. The trailing effect is more marked, and much material is left at the origin. This run was performed with a 4% (w/v) protein sample, dissolved in 75% ethylene glycol and 25% concentrated HCl:KCl buffer. The gel was prepared in the same solvent with 15 g. acrylamide per 100 ml. of solvent. The duration of the run was 19 hr. at 8 v/cm. The pattern shown could not be improved, although all the usual attempts were made.

α-Histone

The electrophoresis of this fraction gave a clear pattern, showing eight bands (Fig. 27). The fastest three bands are well ahead of the following two, which in turn are clearly separated from the intensely staining α-band. Behind this latter, two very faint bands exist in a position which is usually occupied by the bands of the β-group. The same unusual bands

were seen with calf thymus α-fraction (Fig. 19). The trailing effects are slight and some material has been left at the origin. Again the run lasted 19 hr. at a voltage gradient of 8 v/cm. All attempts made to improve the pattern were unsuccessful.

β-Histone

The β-histone gave two poorly separated bands and heavy trailing and shadowing effects (Fig. 27), leaving a great deal of material at the origin. The concentration of protein in the sample used was 4% (w/v), the gel was prepared in 75% ethylene glycol and 25% concentrated HCl:KCl buffer, and the run lasted 19 hr. at 8 v/cm. Once again all attempts to obtain a better resolution of the bands were unsuccessful.

γ-Histone

The γ-histone in its turn gave two bands (Fig. 27). Both bands were sharp and although the whole area was heavily shadowed by strong trailing, the bands could clearly be distinguished because of their intense blue-black colour. Attempts as before to improve on the pattern of this fraction, were once again unsuccessful.

Discussion

The introduction of glycerol and ethylene glycol, as components of the solvents for the preparation of polyacrylamide gels and histone samples used for electrophoresis, presented the problem of the behaviour of these proteins in systems rich in the above solvents. From all the available evidence, described in the Introduction to this chapter, no major conformational changes should be expected to occur within the protein molecule. However, aggregation could not be excluded as it was pointed out by Sage et al. (1962) that ribonuclease is extensively aggregated in ethylene glycol containing potassium chloride. One might then expect the phenomenon of aggregation to occur in histones dissolved in ethylene glycol for the reasons that histones have a high tendency to aggregate anyway, and ribonuclease is, like histones, a basic protein.

The experimental evidence obtained from electrophoretic runs is somewhat conflicting. If a decrease in number of bands of an electrophoretic pattern were an acceptable criterion

of aggregation, then the unfractionated and the β-histones would seem to be in a more aggregated form in organic solvents, (showing fewer bands), and the α and γ-histones would be less aggregated. When histone samples dissolved in 75% ethylene glycol, were run in gels prepared in purely aqueous solvents, the results were roughly the same as those obtained with protein samples dissolved in aqueous media. The number of bands in most cases was the same, and the bands occupied approximately the same positions in the pattern. When histones were dissolved in lanthanum-acetate buffer and this solution put to run in ethylene glycol containing gels the results obtained were very poor, most probably due to the decreased conductivity of the sample. No conclusions can be drawn from this experiment.

The initial observation that the same sample gives different patterns in different gels points towards the gel as the one being responsible for the observed differences.

In general gels prepared in organic solvents do not allow migration as fast as purely aqueous ones. The parameters that seem to be different in the organic solvent gels are the pore size and the internal viscosity. The pore size would be expected to be smaller because of the higher concentration of acrylamide used. This alone does not explain the situation since aqueous gels prepared with a larger amount of acrylamide than usual did not show an increased number of bands in the case of α-histones but instead the migration was shorter and the pattern more 'condensed'.

If the molecular weights of the various histone fractions are taken into account, the situation becomes clearer. The α-fraction consists of many groups with molecular weights in the range 5 to 10 thousand, the unaggregated β-fraction has a molecular weight of the order of 57,000 and the γ-fraction of 74,000 (Cruft et al. 1957b, 1958a, b). The differences between the molecular weights would seem to offer an explanation for the differences in distances migrated. The fact that β and γ-fractions migrate approximately to the same

extent could be explained in part by the relative shapes of the proteins as indicated by the work of Cruft et al. (1958a, b) who found the frictional ratio of the unaggregated β-histone to be 2.5, while that of γ-histone 2.05.

That the increased internal viscosity of the gel, exerts its effects to a different degree with each fraction is a possibility that cannot be excluded. The shapes and sizes of these proteins are not at all clear, and the opinion of different workers varies widely.

It is possible that a denaturation takes place when the histone comes in contact with the organic solvents used although there is no evidence to support it. On the contrary the fact that histone dissolved in 75% ethylene glycol and run in polyacrylamide gel prepared in lanthanum-acetate buffer gave an almost identical pattern to the one given by histone dissolved in aqueous solvent, should be considered evidence against this. It seems more probable that the presence of these high amounts of organic solvent in the solutions of the histones, does not affect their conformational

characteristics, except perhaps in the case of β-histone which is known to aggregate at low pH's, in which case the aggregation would not be attributed to the organic solvent but rather to the low pH buffer.

The resolution obtained with the gels containing organic solvents could be the result of stronger molecular sieving, due to the smaller pore size, together with the differential 'slowing down' effects exerted on the molecules by the high viscosity medium. The fact that the α-histones give the best resolution, supports this assumption, because of their small molecular weights. Furthermore the difference in pore size would explain the presence of more material left at the origin, and the heavy trailing effects that are often noticed in the organic solvent containing gels. Again note might be taken of the fact that the time required by these gels to absorb enough dye from the staining bath is at least four times greater than by the gels prepared in aqueous solvents.

The proposition that these gels have a smaller pore size together with a greater viscosity, is supported by the experiments performed with different acrylamide and organic solvent concentrations. When the gels are prepared with increasing amounts of acrylamide (keeping the organic solvent concentration unchanged) the migration distance decreases steadily and the pattern retains its shape although it is more compact. The number of bands is not seriously affected at least in the cases where the bands could be counted. One might then conclude that the decrease in pore size (which results from the increase in acrylamide concentration) does not affect the resolution, but only shortens the migration distance.

When the acrylamide content of the gels is kept constant and the percentage of organic solvent changed, then the results are unpredictable. In the case where glycerol was used, the gels containing 75% of it in the solvent, the calf thymus α-histones showed a pattern with eleven bands. When the glycerol concentration was reduced to 60% the bands became seven, and

when it was reduced to 50% nine bands appeared. The bands became eleven again when the concentration was 40%, ten when 25% and eleven when 10%.

With gels containing 75% ethylene glycol, the α-histone showed fifteen to seventeen bands. The number of bands were reduced to seven when the ethylene glycol concentration reached 60%, went up to eleven when 50%, down to nine in 40%, eleven in 25% and finally nine again in 10%.

From these experiments the only conclusions that can be drawn is that a simple relationship between resolution and internal viscosity is non-existent. The resolution is definitely affected by the higher viscosity but to an extent that does not seem to follow any clear relation.

The introduction of glycerol and ethylene glycol as components of the solvents in the preparation of the gels was not an unqualified success though was a reasonable improvement over existing methods in some cases. The unfractionated histone either from calf thymus

or rat liver gave better results when run in the 'ordinary' lanthanum-acetate gels. In these gels patterns with thirteen bands for the thymus, and six to seven for the rat-liver histone were obtained; in the gels with glycerol calf thymus histone showed six bands and with ethylene glycol nine bands only. The resolution of unfractionated histone in the aqueous gels was much better than in organic solvent ones.

The α-histone from both sources showed the greatest number of bands of all the fractions in ethylene glycol gels. Whereas nine bands were shown by calf thymus α-histone in aqueous gels, they numbered fifteen to seventeen in ethylene glycol, and eleven in glycerol. Rat liver α-histone from showing five bands in aqueous gels showed eight in ethylene glycol and only four in glycerol.

The β-fractions from the same two sources showed fewer bands in the systems containing the organic solvents, and the γ-fraction although it did give the same number of bands in each system, gave clearer patterns in the gels with the organic solvents.

Histone Fraction	Lanthanum - Acetate Gels		Ethylene glycol Gels		Glycerol Gels	
	Calf Thymus	Rat Liver	Calf Thymus	Rat Liver	Calf Thymus	Rat Liver
whole	13	6-7	9	6	6	3
α	9	5	15 - 17	8	11	4
β	9	4	5	2	3	2
γ	3	2	3	2	3	2

Table I. NUMBERS OF BANDS GIVEN BY HISTONE FRACTIONS RUN IN VARIOUS TYPES OF POLYACRYLAMIDE GEL.

A summary of the results given by the two histone preparations and the histone fractions is shown in Table 1.

The gels prepared in 75% ethylene glycol, with 15 g. of acrylamide per 100 ml. of solvent, were found to be the most satisfactory of all for the electrophoresis of α-histones. They were used routinely whenever this fraction had to be examined and the results have been very satisfactory. The reproducibility was good as far as the number of bands is concerned. Yet in some cases the curving or trailing effects were so pronounced as to give rise to doubt about the pattern. A repeat of the same run usually gave the desired clarity. This bad definition of the pattern was found to be more frequent when the sample was not allowed to 'stand' for a few hours after dissolution. Therefore the samples were usually prepared on the day preceeding their use.

PART III

INVESTIGATIONS INTO THE INCORPORATION OF SCINTILLATORS IN POLYACRYLAMIDE GELS

Introduction

The idea behind the investigations into the use of organic solvents in the preparation of acrylamide gels for histone electrophoresis, was the possibility of incorporating a scintillator into their structure. The advantages of this would be to allow the detection of the histone bands without staining, enable the elution of individual bands for further analysis, and thus the quantitative evaluation of the rate of synthesis of the various fractions would be possible.

The method of ultraviolet light scanning of the gels without staining them does not allow quantitative estimation of the amount of protein in the bands for several reasons. It has been shown (Muecke, 1962) that the different fractions, not unexpectedly, do not show the same correlation between staining and ultraviolet absorption, and though the gels might

first be stained and then scanned in order to examine the relationship, the unknown extent to which light might be absorbed could make this unreliable. Quantitative estimation by staining suffers from the fact that some of the histones colour differently from the rest and similar difficulties are met in ninhydrin staining (Muecke, 1962). The resolution of the scanner would also be poor owing to band curving, trailing effects and the short distances between the bands.

Results and Discussion

The problem of incorporating the scintillator was attacked in two ways. Firstly the copolymerization of styrene and acrylamide, and secondly the polymerization of acrylamide in solvents known to dissolve scintillators were attempted. Styrene was chosen because polystyrene is the simplest of a family of plastic scintillators. Styrene polymerizes by heating at 200°C or by prolonged heating at lower temperatures; it is soluble in ethanol, ether, methanol, acetone and is immiscible with water. Because of this immiscibility with water the only solvent tried was ethanol as it

is the only one that dissolves both styrene and all the chemicals needed for the preparation of the gels.

A 10% acrylamide solution (containing N,N'-methylenebisacrylamide, the catalyst and the ammonium persulphate) was prepared in a 90% ethanol, and 10% 0.2 M acetate buffer pH 4.2 solution. Twenty millilitres of the above solution were poured into each of twelve tubes, and styrene added in volumes of 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 10, 15, and 20 ml. per tube. Immediately after the addition of styrene all the tubes were clear and the solutions homogeneous. The samples were allowed to stand at room temperature. After about 30 min. the tube with the highest concentration of styrene showed a white precipitate and gradually the rest followed. The precipitate was found to be soluble in water.

The same solutions as above were again prepared and after the addition of styrene were heated to 80°C for various lengths of time up to 10 hr. The solutions remained clear while warm, but showed the same white precipitate on cooling.

SOLVENT	SOLUBILITY					
	Alcohol [1]	Ether [1]	Water [1]	Ethylene glycol & Glycerol	Acetate Buffer [2]	RPH [3]
Dioxan	∞	∞	∞	miscible	miscible	70
Anisole	s	s	i	i	i	100
Ethylbenzene	∞	∞	.14	i	i	96
Mesitylene	s	s	i	i	i	82
p-Cymene	vs	s	i	i	i	80
Toluene	∞	∞	.47	i	i	100
Xylene	∞	∞	i	i	i	107
Phenyl-cyclohexane	vs	vs	i	i	i	102

Table 2. SOME CHARACTERISTICS OF THE PRINCIPAL SCINTILLATOR SOLVENTS.

1. Data taken from the "Handbook of Chemistry and Physics".
2. Containing in solution the constituents of polyacrylamide gel.
3. RPH: Relative Pulse Height, refered to toluene containing 3g/lit PPO as 100. Taken from Hayes et. al. (1955).
s: soluble, i: insoluble (immiscible), vs: very soluble.

It seems that the product of polymerization of acrylamide is insoluble in alcohol and precipitates as soon as it is formed since when it was collected by centrifugation and dissolved in water a gel was produced within a few hours.

Experiments were therefore undertaken to find out if the acrylamide could polymerize in solvents capable of dissolving scintillators. The scintillators chosen were diphenyloxazol (PPO) and 1,4-di- 2-(5-phenyloxazolyl) -benzene (POPOP), because they are constituents of all liquid scintillation mixtures that are capable of holding a certain amount of water. The most common solvents for PPO and POPOP together with some of their properties are listed in Table 2.

By comparing the various solvents it became clear that the only one that could be used was dioxan, being miscible with water, the solution for the preparation of polyacrylamide gels, and ethylene glycol.

Gels were prepared in up to 90% dioxan but all of them although they polymerized became white, harder than usual and very easily broken. Presumably the polyacrylamide structure collapses in dioxan.

An attempt was then made to prepare the gel in a mixture consisting of one part xylene, 3 parts dioxan, and 3 parts cellosolve (Schramm, 1963). This mixture is capable of holding 29.2% water and its efficiency for detecting carbon-14 is 46.5%. The results however were the same as before.

Other mixtures tried were, methanol-ethylene glycol-dioxan (Bray, 1960), the dioxan based NE 220 liquid scintillator (Nuclear Enterprises, Ltd.), and finally dioxan-anisole-1,2-dimethoxyethane which is especially good for holding ethylene glycol in solution (Davidson, 1958). None of the above mixtures of solvents yielded a clear gel. All turned out to be hard, white and brittle.

As the experiments intended for the polymerization of acrylamide in scintillator solvents failed, the possibility of incorporating the solvents by diffusion after the gel had polymerized, was investigated.

Gels were prepared in purely aqueous, ethylene glycol, and glycerol mixtures and each was dipped in every one of the solvents shown in Table 2. There was no difference between

the gels prepared in purely aqueous media and those prepared with organic solvents. The gels showed no large changes in appearance after being treated with these scintillator solvents, (except dioxan), becoming slightly harder but remaining transparent. In contrast the gels put into the dioxan bath became white and hard.

In order to find out whether the solvents penetrate the gel structure, the experiments were repeated with solutions of the scintillators in the same solvents. The concentration of the scintillators was 3 g./lit. of PPO and 0.3 g./lit. of POPOP. The gels were left in contact with scintillation solutions for up to one week. On the surface of the gels appeared a deposit that made them look slightly opaque, and they scintillated strongly when exposed to ultraviolet light. These gels were then cut across and the newly cut surfaces, exposed to ultraviolet light. The scintillation this time emanated only from the top surface of the gels, while the inner parts of the gels did not scintillate at all.

From this observation it was concluded that the solutions did not penetrate the gel structure, but were only adsorbed onto the exposed surfaces.

At this point these series of experiments were discontinued.

CHAPTER TWO

PREPARATION AND FRACTIONATION OF HISTONES

A brief account of the methods applied to the extraction and fractionation of histones has already been given in the Introduction to this thesis. These methods differ widely and so do their results. It was therefore considered desirable to repeat each method individually and check the results by using polyacrylamide gel electrophoresis.

The methods that used whole tissues for the extraction of histones were excluded in order to avoid any possible contamination with ribosomal basic proteins. The effect of these proteins on the electrophoretic pattern of histones is not known and probable interference might have been attributed to other causes.

The methods used are:

1. The isolation of nuclei with 4% acetic acid (Stedman and Stedman, 1951; Cruft et al. 1957a).
2. The extraction of histones with dilute acids (Stedman et al. 1951; Bakay et al. 1955).
3. The ethanol chemical fractionation of Cruft et al. (1957b, 1958b).

4. The fractionation of histone using carboxymethyl-cellulose chromatography (Johns,1963)
5. The extraction of α-histones with perchloric acid (Johns and Butler, 1962).
6. The extraction of β-histones with ethanol-hydrochloric acid mixture (Johns et al. 1960).
7. The fractional precipitation of α-histones with trichloroacetic acid (Johns, 1963).
8. The use of Amberlite IRC-50 ion exchange resin, for the fractionation of an α-histone subfraction (Johns, 1963).

ISOLATION OF CELL NUCLEI

The method that has always been applied in the preparation of nuclei was that described by Stedman et al. (1951) as modified by Cruft et al. (1957b). Most of the work was done with calf thymus glands and the technique was applied as published. For the isolation of rat liver nuclei there was a slight modification which is mentioned appropriately.

Preparation of calf thymus nuclei

Fresh tissues were removed from the carcasses immediately after the killing of the animals in the slaughter house, and transported in crushed ice. The operations started within one hour of the death of the animals and were always carried out at 2 - 4°C. either in the cold room or in a refrigerated centrifuge.

The glands were at first cleaned of as much fat and connective tissue as possible. They were then passed through a coarse household mincer and the resulting pulp was forced through a fine extrusion mincer. The pulp was suspended in two volumes of 4% (v/v) acetic acid previously chilled to 2°C and the mixture

allowed to stand overnight, in the cold room. The pH of the mixture ranged from 3.6 to 3.9. The overnight pulp was then stirred rapidly with a motor driven blade which is surrounded by a set of stationary spikes. The spikes collected a large amount of fibrous tissue which was withdrawn from time to time. The material was then filtered through four layers of muslin and centrifuged at 1000 g for 15 min. in a refrigerated centrifuge, to sediment the nuclei. The nuclei came out well packed. On the top there was a thin layer of brownish material composed of cell debris and fragments. This was removed along with the supernatant. The nuclei were then suspended in six volumes of 1% chilled acetic acid, mixed for about 2 min. with a mechanical stirrer and centrifuged. The buff material on top of the nuclei was again removed and further similar washings with 1% acetic acid were performed. In all, the nuclei were washed approximately eight times before a preparation of clean nuclei, free of whole cells or cytoplasmic material, was obtained.

The thin layer of brown material is usually present on top of the sedimented nuclei for the first four washes. After the last wash with 1% acetic adid the nuclei were suspended in chilled ethanol, centrifuged at 1000 g and resuspended in ethanol. This time they were left in the refrigerator overnight and then centrifuged before washing once more with ethanol. This was followed by two ether washes, and finally after centrifuging at 1500 g for 30 min. the nuclei were left to dry in air.

The yield of dry nuclei was between 4 and 4.5% of the weight of wet tissue.

Preparation of rat liver nuclei

For the preparation of nuclei from rat liver it was found necessary to homogenize the tissue because the existing mincing equipment was too large for the amount of material available.

Tissues were removed from the rats immediately after death, and cleaned of connective tissue with a pair of scissors. They were then put into 4% chilled acetic acid and cut

into very small pieces, first with scissors and then with a razor blade. This treatment yielded a very thick pulp which was homogenized in a glass homogenizer with a Teflon piston (clearance approximately 0.2 mm.). Only four strokes of the piston were necessary. The preparation was then continued as described for the calf thymus, except that the stage of using the motor driven blades with the stationary spikes for the collection of connective tissue, was omitted, as it was not found necessary.

The yield of dry nuclei from rat liver varied from 0.6 to 0.93% of the weight of wet tissue.

However, in the case of regenerating rat liver nuclei, it was found to be more constant and of the order of 1%.

Comment

The nuclei obtained by the method described were examined following each preparation, by viewing them under the microscope after staining with methylene blue.

Nuclei derived from thymus glands have always been found to be extremely pure and the presence of any contamination was very rare.

In the case of rat liver nuclei the preparations were never as pure. It was found that for every hundred clean nuclei, there were five to ten whole cells, or nuclei having some cytoplasm adhering to their surface.

In the case of nuclei from regenerating rat liver however, this figure was at least halved, and the nuclei were much cleaner.

EXTRACTION OF HISTONES

The histones have in all cases been extracted from isolated nuclei, with 0.1 N hydrochloric or sulphuric acid. The difference in yield between the two acids was found to be negligible. The factor that dictated which one was to be used, was the purpose for which the histones were extracted. If the histones were to be fractionated with ethanol, then histone sulphate was necessary; if it were to be prepared in ethylene glycol solution, then histone chloride was needed. However, the transition from sulphate to chloride or vice versa has also been used successfully. This was done by exhaustive dialysis first against water and then against a 0.1 N solution of the acid whose salt was required.

The dry nuclei, usually in batches of 5 g. were moistened with ethanol and suspended uniformly in distilled water. The water was removed by sedimenting the nuclei at 2500 g for 20 min. The acid solution was then added (10 ml./g. nuclei) and the mixture stirred for 5 min. It was left for approximately 2 hr.

ORGAN	NUCLEI °/o *	HISTONE - HCl		HISTONE - H_2SO_4	
		°/o OF NUCLEI	°/o OF WET TISSUE	°/o OF NUCLEI	°/o OF WET TISSUE
CALF THYMUS X	4.2 (3.8 - 4.5)	25.6 (23 - 26.2) VI	1.03 (.9 - 1.1) VI	25.8 (21 - 28) IV	1.08 (.99 - 1.15) IV
RAT LIVER VI	.786 (.6 - .93)	26.4 (24.4 - 29)	.215 (.19 - .25)	—	—
REGENERATING RAT LIVER	.964 (.57 - 1.1)	15.2 (13 - 17.9)	.143 (.17 - .13)	—	—

Table 3. YIELDS OF NUCLEI AND HISTONES.

* The percentage of nuclei is given as weight of dry nuclei per 100g of wet tissue.
The Roman figures denote the number of preparations.
The figures in parentheses represent the ranges of values obtained.

at room temperature with occasional stirring. Centrifugation was then performed to pack the nuclei and collect the supernatant. This latter was kept aside in the refrigerator, and to it the supernatants of the successive extractions were added. The second extraction was performed in the same way, except that it was left overnight; the third was done for a period of about 6 hr. and the fourth for the same period. This last extraction yielded only traces of histone, if any at all.

The joint supernatants were treated as follows: in the case of sulphuric acid extracts the histones were precipitated with seven volumes of absolute ethanol, and in the case of hydrochloric acid extracts by exhaustive dialysis against acetone. In both cases the precipitates were washed twice with acetone, twice with ether and were air dried.

In Table 3 the yields of nuclei and histones from the organs used for extraction, are summarized.

Comment

From the results given in Table 3 it can be appreciated immediately that the difference in yield between sulphuric and hydrochloric acid extractions was virtually nil.

Also worthy of mention is the enormous difference in the yield of nuclei and histones from the two different tissues.

The results concerning the rat tissues were treated statistically. The histone contents of normal and regenerating rat liver were found to be different (significant to the 0.1% level). The content of nuclei in the normal and regenerating rat liver tissue were different only at the 10% level. However, from seven preparations of regenerating rat liver nuclei six were in the range 0.95 to 1.1, and the seventh was 0.57. The standard deviation (SD) of the mean value (0.964), is 0.167 and less than 0.95% of all observations lie more than 2.35 x (SD) outside the mean value (mean - 2.35 x (SD) = 0.571).

Considering that the value 0.57 may be disregarded and treating the remaining six statistically, the difference between nuclei content of normal and regenerating rat liver, lay at the 0.1% level of significance also.

From the results of Table 3 it is obvious that in the regenerating rat liver the amount of cell nuclei is higher by 22.6% than in normal rat liver, and the histone content of the nuclei is lower by 41.7%.

These results correspond with those of Cruft *et al.* (1957a) for the cases of normal rat liver and hepatoma. According to the figures given by the above workers, histone-sulphate constitutes 22% of the nuclei of normal liver and 16.8% of nuclei extracted from rat hepatoma. This finding is in agreement with Stedman's theory according to which rapidly dividing cells contain less histone (Stedman *et al.* 1951).

FRACTIONATION OF HISTONES

Ethanol precipitation method

Application to calf thymus histone

The method used was the one originally published by Cruft et al. (1957b, 1958b) with some modifications.

Twenty grams of whole histone sulphate were dissolved in 500 ml. of water (4% solution). The solution was centrifuged at 6,000 g in an M.S.E. preparative centrifuge to get rid of insoluble material and impurities, and the pH was adjusted to 10 with a concentrated (1 g./ml.) solution of sodium hydroxide. This produced a yellowsih colour but the solution remained clear. Ethanol was then added to a final concentration of 17% and kept at 4°C. overnight. This preparation was then centrifuged at 5,000 g for 40 min. when the precipitate formed was well packed and the supernatant absolutely clear. The precipitate contained the β and γ-fractions and the supernatant the α-fraction.

The supernatant was dialyzed for 2 days against distilled water, followed by 0.1 M hydrochloric acid for 4 days, by which time the

dialysis bath showed no sulphates present. The bag contents were then dialyzed against acetone for 2 days, taken from the dialysis tubing, washed with acetone and ether (twice with each) and air dried. The yield was 4.5 g. or 21.4% of the starting material. (It should be noted that the product is in the chloride form while the starting material was in the sulphate form. This yield may therefore be slightly low).

The precipitate was dissolved in 600 ml. of 0.1 M hydrochloric acid and dialyzed against distilled water for a period of 4 days to remove the sulphates. The pH was then adjusted to 9.5 with 7.5 ml. of 25% (w/v) sodium hydroxide solution, and the ionic strength adjusted to one by adding 32.07 g. of sodium chloride. The solution was left at room temperature for 3 days. A gelatinous suspension formed and was precipitated by centrifuging for 2 hr. at 6,500 g. The precipitate contained the β-histones and the supernatant the γ .

The supernatant was dialyzed against glycine-sodium hydroxide buffer pH 10 for 4 days with feequent changes of the dialysis bath.

The final volume was 680 ml. and the pH 10. Ethanol was then added to bring the volume up to 820 ml. (17% in ethanol). A white precipitate formed immediately and was packed by centrifugation for 1 hr. at 1,500 g. After removing the supernatant the precipitate was dissolved in 200 ml. of 0.1 M hydrochloric acid, the pH adjusted with 25% (w/v) sodium hydroxide to ten, and ethanol added to a final concentration of 17%. This was left for 3 hr. at room temperature and centrifuged at 1,500 g for one hour. The white precipitate was collected and dissolved in 300 ml. 0.1 M hydrochloric acid. The pH was adjusted to 5 with sodium hydroxide solution and the ionic strength to three with sodium chloride. This was left for 5 days at room temperature, and then centrifuged at 65,000 g for 1 hr. The precipitate (consisting of small amounts of impurities from other fractions) was rejected and the supernatant dialyzed exhaustively against water to remove the salt. The dialysis bath was then changed to 0.1 M hydrochloric acid, for 1 day, followed by acetone for two days. The predipitate (γ-histone) was collected, washed twice with

acetone, then twice with ether and air dried. The yield was 8.9 g. or 44.5% of the starting material.

The precipitate, containing the β-histones, was dissolved in 300 ml. of 0.1 M hydrochloric acid. The pH was adjusted to five with sodium hydroxide and 52.65 g. of sodium chloride was added to bring the ionic strength up to 3. The solution was left at room temperature overnight and then centrifuged at 2,500 g for 1 hr. This treatment was repeated four times in all and the last time the solution was centrifuged at 65,000 g for 1 hr., the precipitate redissolved in 300 ml. of 0.1 M hydrochloric acid, dialyzed exhaustively against the same solvent at first, and finally against acetone. The β-histones were collected, washed with acetone, then ether and air dried.

The yield was 5.7 g. or 28.5% of the starting material.

Comment

The differences between the method just described and that originally published lie firstly in the time allowed for the β-histone

to aggregate while in solution with the γ-fraction (three days instead of no time at all), and secondly in that the histone fractions were converted to chlorides and precipitated with acetone, instead of sulphates precipitated with ethanol.

The β-fraction was found to be very pure, and there was never any trace of β-histones in the γ-fraction. This increased purity is attributed to the time allowed for the aggregation. Electrophoresis of the above three fractions was done and the photographs of the results are those included in the first section (Figs. 4, 5, 6 and 7).

At the stage of the first precipitation of the γ-histones the supernatant was kept, as it was suggested by Cruft and co-workers originally, that it contained some α-histones. This was dialyzed for four days against 0.1 M HCl followed by dialysis against acetone. The white precipitate collected after the centrifugation was examined electrophoretically and found to be an impure α-fraction and as its weight was only 500 mg. no attempts to fractionate it were made. A summary of the method used follows on page 114.

-114-

WHOLE HISTONE

Dissolved in water, clarified by centrifugation, pH adjusted to 10 with NaOH, Ethanol added up to 17%. Kept overnight. Centrifuged at 6000 g. for 40 min.

Precipitate

β and γ-histones

Dissolved in 0.1 M HCl, dialyzed against water pH adjusted to 9.5 (with NaOH), $\frac{1}{2}\Gamma$=1 (with NaCl) Left for 3 days at room temperature. Centrifuged at 65,000 g.

Precipitate

β-histones

Dissolved in 0.1 M HCl, pH adjusted to 5 (with NaOH), $\frac{1}{2}\Gamma$= 3 (with NaCl). Left at room temperature overnight. Centrifuged (Repeated four times).

Precipitate

Dissolved in 0.1 M HCl. Dialyzed against 0.1 M HCl followed by acetone. Washed with acetone and ether. Air dried.

β-histones

Supernatant

Rejected

Supernatant

α-histones

Dialyzed against water, then 0.1 M HCl and acetone. Washed with acetone and ether. Air dried.

α-histones

Supernatant

γ-histones

Dialyzed against buffer pH 10. Ethanol added to give final concentration of 17%. Centrifuged at 1500 g.

Precipitate

γ-histone

Dissolved in 0.1 M HCl, pH adjusted to 10 (with NaOH), ethanol added to give a final concentration of 17%. Left for 3 hr. at room temperature Centrifuged at 1500 g. for 60 min.

Precipitate

Dissolved in 0.1 M HCl pH adjusted to 5 with NaOH, $\frac{1}{2}\Gamma$= 3 with NaCl Left for 5 days at room temperature. Centrifuged at 65,000 g for 1 hr.

Precipitate

Rejected

Supernatant

checked for α's.

Supernatant

Rejected

Supernatant

Dialyzed against water, 0.1 M HCl and acetone. Washed with acetone and ether. Air dried

-histones

Fractionation of rat liver histones

The ethanol precipitation technique was applied to the fractionation of rat liver histones. The differences observed during the procedure were firstly the large amount of protein insoluble in water, and secondly the poor yield. While with calf thymus histone the water insoluble material was present only in trace amounts, with rat liver histone there was almost 15%. Electrophoresis of the insoluble part in polyacrylamide gel yielded a smear in which no bands were resolved, and left a considerable quantity of material in the origin.

The yields of fractions from rat liver were:

α-histone, 10.5% of the whole histone

β-histone, 23.9% of the whole histone

γ-histone, 16.2% of the whole histone

Total yield, 50.6% of the whole histone

The 15% insoluble material has been excluded from the calculation of the yields.

Photographs of the electrophoretic runs of the above fractions are the ones shown in Figs. 11, 12 and 13. Note: when the amount of material available was less than 100 mg. the yields of this fractionation technique were poor (25 to 30%).

Comment

The differences between the calf thymus histones and those extracted from rat liver do not only lie in the electrophoretic pattern. Apart from the large amount of rat liver material insoluble in water, already mentioned, differences were observed during the application of the fractionation procedure and in the handling of these proteins. The precipitates formed as a rule more quickly and the solution in all the solvents used was much more difficult. When hydrochloric acid was the solvent there was little difference between the samples, but when they were prepared in lanthanum-acetate buffer pH 4.9 for polyacrylamide gel electrophoresis, there was a lot of insoluble material left, which dissolved slowly, only when the pH was lowered to 3 by the addition of glacial acetic acid.

The aggregation of histones from rat liver seems to be more sensitive to environment than that of histones from calf thymus. The calf thymus-histone solutions, provided the pH is of the order of 3, can be kept at room temperature

for several weeks without any noticeable aggregation taking place. The same did not apply to rat liver histones, as after a few days the solutions had to be rejected and replaced with fresh ones. Keeping them in the refrigerator did not help.

Disaggregation was also found to be different. Solutions of calf thymus histones disaggregated immediately on solution (there were some exceptions). The solutions of rat liver histones required to stand approximately one day before disaggregation diminished. This was checked by polyacrylamide gel electrophoresis.

EXTRACTION OF α-HISTONES WITH PERCHLORIC ACID

The first of the methods tried for the fractional extraction of histones was the one reported by Johns (1963). It was applied by him to the extraction of the very lysine-rich histones, directly from tissue homogenates. As the whole tissue was involved originally, it was modified and used with nuclei, whole histone and α-histone. In all cases the concentration of perchloric acid was 5%, the one originally published by Johns.

Extraction of calf thymus nuclei

Five grams of nuclei were wetted with a few drops of ethanol, water was added to form a suspension and this was then centrifuged. The supernatant was rejected and the packed nuclei extracted with 10 ml. of 5% perchloric acid for 24 hr. at 4°C. They were then centrifuged at 1,500 g for 20 min., the supernatant kept and the nuclei re-extracted in the same way twice more. (The third extraction showed no precipitate with trichloroacetic acid). The three joint supernatants were dialyzed for 3 days against 0.1 M hydrochloric acid, followed

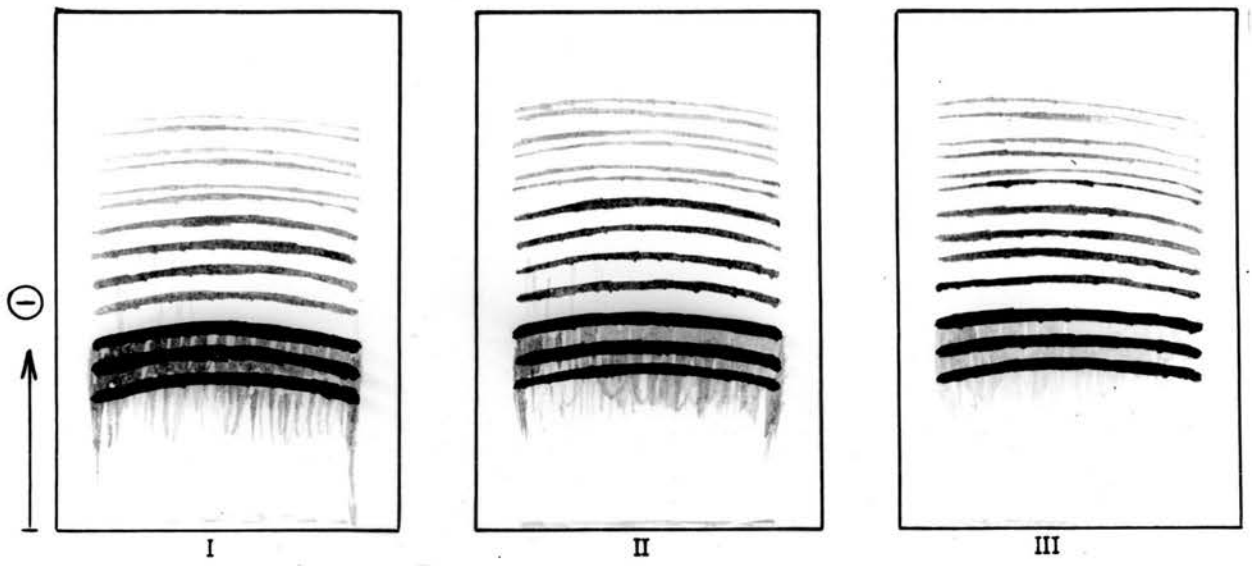


Fig. 28A. ELECTROPHORETIC PATTERNS IN ETHYLENE GLYCOL POLYACRYLAMIDE GELS OF EXTRACTIONS OF a-HISTONES WITH 5 °/o PERCHLORIC ACID. I: FROM NUCLEI, II: FROM WHOLE HISTONE, III: FROM a-HISTONE.

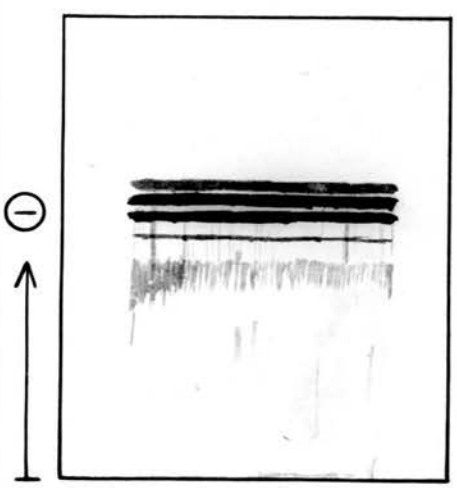


Fig. 28B. ELECTROPHORETIC PATTERN OF THE RESIDUE OF THE WHOLE HISTONE EXTRACTION OBTAINED WITH A LANTHANUM ACETATE GEL.

by dialysis against acetone. The precipitate was removed from the dialysis tubing, centrifuged and washed twice with acetone and again twice with ether. It was air dried. The dry weight was 208 mg. or 4.16% of the weight of the nuclei.

The α-histone isolated in the above way was examined by polyacrylamide gel electrophoresis, in a gel containing 75% ethylene glycol. It was found to be a good preparation showing the same number of bands as the α-histone obtained otherwise (Fig. 28).

Extraction of calf thymus whole histone

Two hundred milligrams of whole histone-HCl were extracted twice with 5% (v/v) perchloric acid at 4°C for 24 hr. The two supernatants were mixed together and dialyzed against 0.1 M hydrochloric acid followed by dialysis against acetone. The precipitate was collected, washed with acetone, ether and air dried, yielding 25 mg. or 12.5% of the whole histone.

The residue of the extraction was dissolved in 20 ml. of 0.1 M hydrochloric acid, and dialyzed against the same solvent at first and

against acetone later. The precipitate formed was centrifuged, washed with acetone and with ether before being air dried. The weight was 155 mg. or 77.7% of the whole histone.

The recovery of the two fractions was 90.2%.

From the electrophoretic runs in acrylamide gel, containing ethylene glycol (Fig. 28A) for the first fraction (α-histones), and lanthanum-acetate buffer for the second (β and γ-histones), shown in Fig. 28B, it was seen that the α-fraction was pure and gave good electrophoretic patterns, but the β and γ-fractions showed patterns with fewer bands and considerable diffusion.

The above procedure was repeated with histone-H_2SO_4. The yields from 250 mg. of histone were 40 mg. of α-histone (16%) and 205 mg. of β plus γ-histones (82%) giving an overall recovery of 98% which is better than the 90.2% obtained with histone-HCl. The purity of the fractions was the same as in the previous case.

Extraction of calf thymus α-histone

Two hundred and fifty milligrams of α-histone, prepared by the ethanol fractionation technique, were dissolved in 20 ml. 5% (v/v) perchloric acid and left at 4°C. overnight. The solution was then centrifuged at 6,000 g for 1 hr. A slight precipitate had been formed, which looked brownish and was rejected. The supernatant was dialyzed exhaustively against 0.1 M hydrochloric acid, and then acetone, centrifuged to collect the precipitate which was washed with acetone and with ether and allowed to dry in the air. The dry weight was 210 mg. and the recovery 82%.

This α-histone fraction when compared in polyacrylamide gel electrophoresis with the untreated one showed no differences at all, the two samples giving identical patterns.

Extraction of rat liver whole histone

Rat liver whole histone extracted in the same way as calf thymus histone with 5% perchloric acid, yielded only traces of a white precipitate. The amount of this precipitate was very small (of the order of 1 mg. out of

50 mg. of starting material), and it was not possible to check it electrophoretically. The remaining insoluble material had acquired a yellowish colour, and when treated with lanthanum-acetate buffer pH 4.9 and acidified with glacial acetic acid plenty of insoluble material was still left. Insoluble material was also left when 0.1 M hydrochloric acid was the solvent used. Electrophoretic runs showed the complete absence of α's and no information could be obtained about the β and γ-fraction, as all the bands were very broad and diffused.

Comment

The application of the perchloric acid property of specifically dissolving the α-histones proved to be a quick and reliable method at least for calf thymus histone. When used to extract either protein, or nuclei, the results were very good. The product was in all cases pure as judged by the pattern it gave in electrophoresis. However if one is interested in the remaining β and γ-fractions then this method cannot be recommended.

The electrophoretic patterns obtained with the β and γ-fraction (left after the extraction of the α's), showed very few bands (differing from one preparation to another), intense staining due to material left at the origin and much diffusion on the gel.

Rat liver β and γ-fraction seem certainly to be denatured since they were not completely soluble even in 0.1 M hydrochloric acid, in which they had been extracted from the nuclei and all attempts made to obtain clear electrophoretic pattern failed.

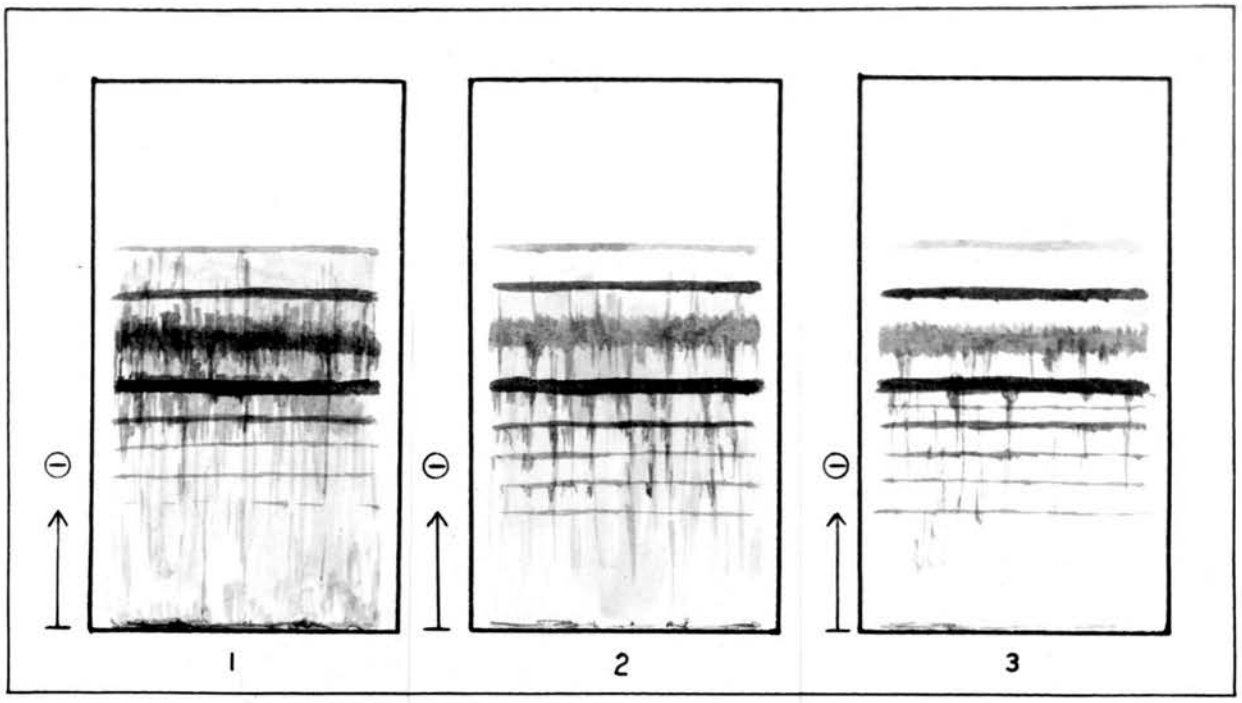


Fig. 29 POLYACRYLAMIDE GEL ELECTROPHORESIS OF β-HISTONE EXTRACTED WITH ETHANOL-HCl

1 EXTRACTION OF CALF THYMUS NUCLEI

2 EXTRACTION OF CALF THYMUS WHOLE HISTONE

3 EXTRACTION OF CALF THYMUS β-HISTONE

EXTRACTION OF β-HISTONES WITH ETHANOL-HYDROCHLORIC ACID MIXTURE

This technique was applied by Johns et al. (1960) to the extraction of the arginine-rich histones from calf thymus tissue homogenate. As the whole tissue was involved the method was again modified and applied to isolated nuclei, whole histone and β-histone. The solvent used was ethanol - 1.25 N hydrochloric acid (80 : 20 v/v).

Extraction of nuclei

Five hundred milligrams of nuclei were extracted three times with 30 ml. of ethanol - 1.25 N hydrochloric acid (80 : 20 v/v). Each extraction was allowed to proceed overnight at 4°C. The supernatants after centrifugation were put together in a dialysis bag and dialyzed against 0.1 M hydrochloric acid, and the extract was precipitated by dialysis against acetone. The precipitate was washed with acetone, ether and air dried. The yield was 35.6 mg. or 7.12% of the nuclei (w/v).

When the β-histone, obtained by the procedure just described, was tested by electrophoresis in polacrylamide gel, it was found to give the usual pattern of β-histone (Fig. 29).

Extraction of whole histone

Two hundred milligrams of whole histone-hydrochloride were extracted three times with 30 ml. of ethanol - 1.25 N hydrochloric acid (80 : 20 v/v). The extractions were carried out overnight at 4°C. The joint supernatants were treated as in the case of nuclei above. The yield of β-histone was 48 mg. or 24% of the whole histone. Again this preparation was found not to differ from the other β-histone preparations, when examined electrophoretically (Fig. 29). Yet the remaining histone which consists of the α and γ-fractions, when examined electrophoretically did not give a clear pattern, and showed very intense trailing effect and a lot of material remaining on the starting line without penetrating the gel.

Extraction of β-histone

The β-histone used for this extraction was prepared by the ethanol chemical fractionation. Two hundred milligrams were extracted three times with 30 ml. of the ethanol - 1.25 hydrochloric acid mixture, as has already been

described. Three extractions were performed because there was an insoluble residue after the first extraction. The supernatants of the centrifugations were put together and the β-histone recovered by dialysis as previously mentioned.

The insoluble material was dissolved in 0.1 M hydrochloric acid, dialyzed against the same solvent at first, and later against acetone. The precipitate formed was recovered by centrifugation, washed with acetone and ether and air dried. The yield of extracted β-histone was 154 mg. (76% of the initial), and that of the residue 14 mg. (7% of the initial).

The extracted β-histone when examined by polyacrylamide gel electrophoresis was found to give the clear distinctive bands given by the other β preparations (Fig. 29).

The insoluble material gave somewhat confusing patterns. A great deal of aggregated material was left in the starting line and there were no sharp bands for identification. Most probably it consisted of impurities from α and γ-fractions, as it showed some very fast moving

turquoise staining material, and a blue-black very diffused zone, in the place usually occupied by the dark staining γ-bands.

Comment

The isolation of β-histone with ethanol - hydrochloric acid proved to be a good method. It yielded very pure fractions with less aggregated material than usual. The yields are somewhat lower than those of the ethanol precipitation technique and can most certainly be successfully applied for the purification of impure β-fractions.

This fractional extraction is a useful one provided the remaining α and γ-fractions are not to be used since the electrophoretic patterns obtained with these fractions were not clear at all, showing intense trailing effects and much aggregated material left at the origin. This could be caused by some material of the β-fraction not being extracted by the ethanol-hydrochloric acid mixture.

FRACTIONATION OF HISTONES ON CM-CELLULOSE CHROMATOGRAPHY

This technique was applied by Johns (1963) for the fractionation of calf thymus whole histone. It was repeated in order to duplicate the results and compare the fractions obtained, and it was also applied for the fractionation of calf thymus α-histone and whole histones from normal and regenerating rat liver.

Fractionation of calf thymus whole histone

Two hundred milligrams of whole histone were dissolved in 40 ml. of acetate buffer pH 4.2 containing 4.07 g. sodium acetate and 70 ml. N acetic acid in 1 lit. The solution was applied to a CM-cellulose column containing 10 g. of Whatman No. 70 CM-cellulose powder (2.5 x 20 cm.). The column had been previously washed with 0.1 N sodium hydroxide, 0.1N hydrochloric acid and equilibrated with the above buffer.

The elution was carried out in three steps. The first eluant was 250 ml. of a solution containing 7 g. of sodium acetate, 120 ml. of 1 N acetic acid, and 24.5 g. sodium chloride in

1 lit., referred to as solution A in the elution patterns. This was followed by 250 ml. of 0.01 N hydrochloric acid, and 250 ml. of 0.02 N hydrochloric acid. The flow rate was kept at 1 ml./min. and fractions of 5 ml. were collected in a fraction collector. The progress of the elution was followed by measuring the optical extinction of each fraction at 278 mμ. The results are shown on the graph of Fig. 30.

The fractions 5 - 20 were mixed together, as were the fractions 52 - 70 and 105 - 125. These were poured into three dialysis bags, and the one containing the fractions 5 - 20 was dialyzed exhaustively against 0.1 M HCl, followed by dialysis against acetone. The other two bags containing the fractions 52 - 70 and 105 - 125 respectively were dialyzed directly against acetone. The precipitates formed were collected by centrifugation, washed with acetone and ether and air dried. The yields were:

Fractions 5-20, 30 mg. or 15% of starting material
Fractions 52-70, 46 mg. or 23% of starting material
Fractions 105-125, 38 mg. or 19% of starting material

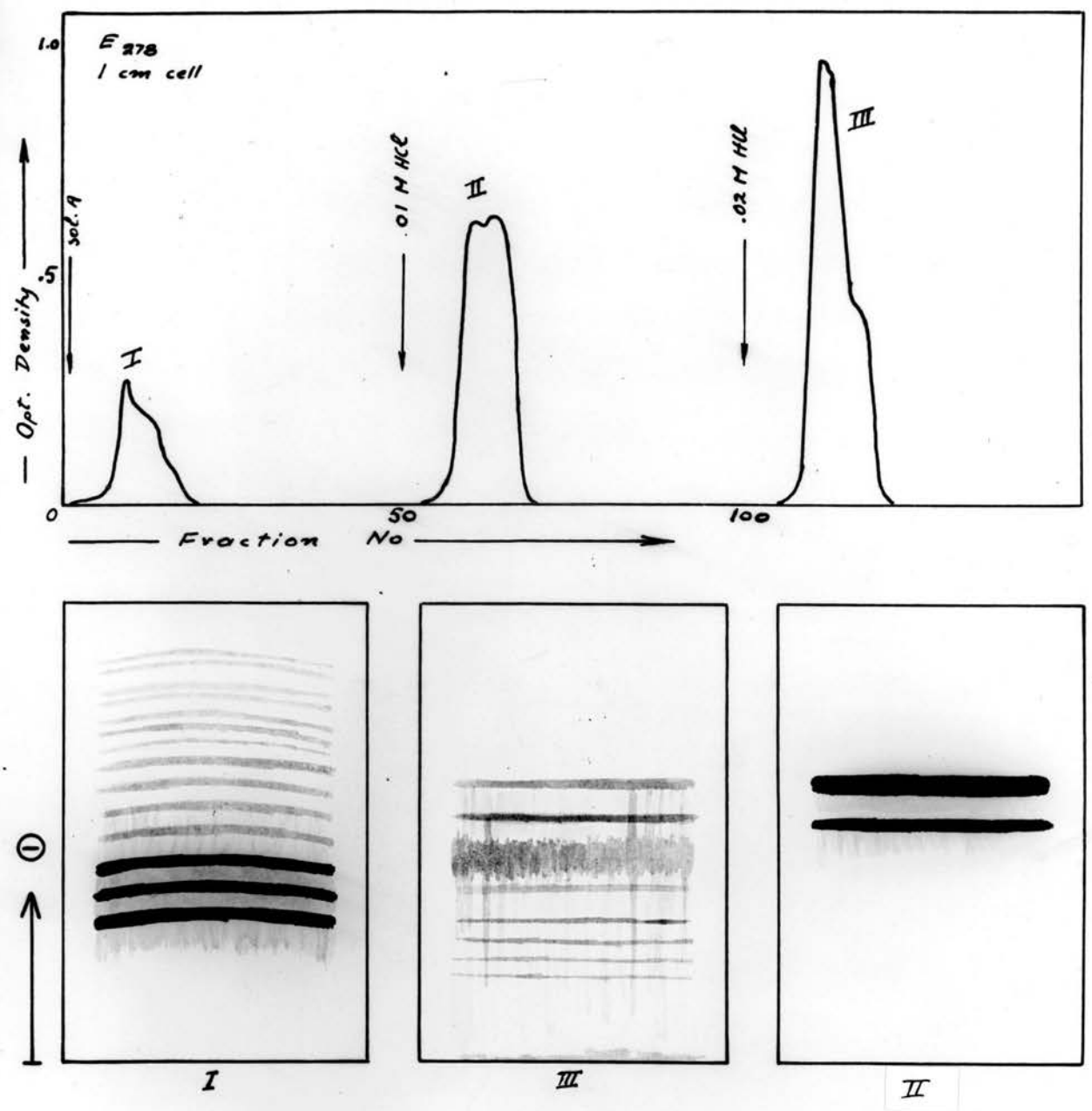


Fig. 30. CM-CELLULOSE CHROMATOGRAPHY OF CALF THYMUS HISTONE AND ELECTROPHORETIC PATTERNS OF THE FRACTIONS.
I: GEL CONTAINING 75% ETHYLENE GLYCOL
II & III GELS PREPARED IN LANTHANUM-ACETATE.

These fractions were examined by polyacrylamide gel electrophoresis and were found to be typical examples of α, γ, and β respectively (Fig. 30).

The yield however was much less (57%) than that claimed by Johns (80%), and than that obtained by the ethanol precipitation procedure (94%). The considerable advantages of the method are its speed, and its good reproducibility.

Fractionation of α-histones in CM-cellulose

This experiment was undertaken in the hope that α-histones might yield subfractions. The procedure followed was identical to that described for whole calf thymus histone. The amount of α-histone applied on the column was 500 mg. It was eluted stepwise with the same three solvents as above, and the histone was recovered by conversion of the chloride salt and dialysis against acetone. The results are shown on the graph in Fig. 31. The fractions 5 - 35 were put together in a dialysis bag, and dialyzed against 0.1 M hydrochloric acid at first, followed by dialysis against acetone.

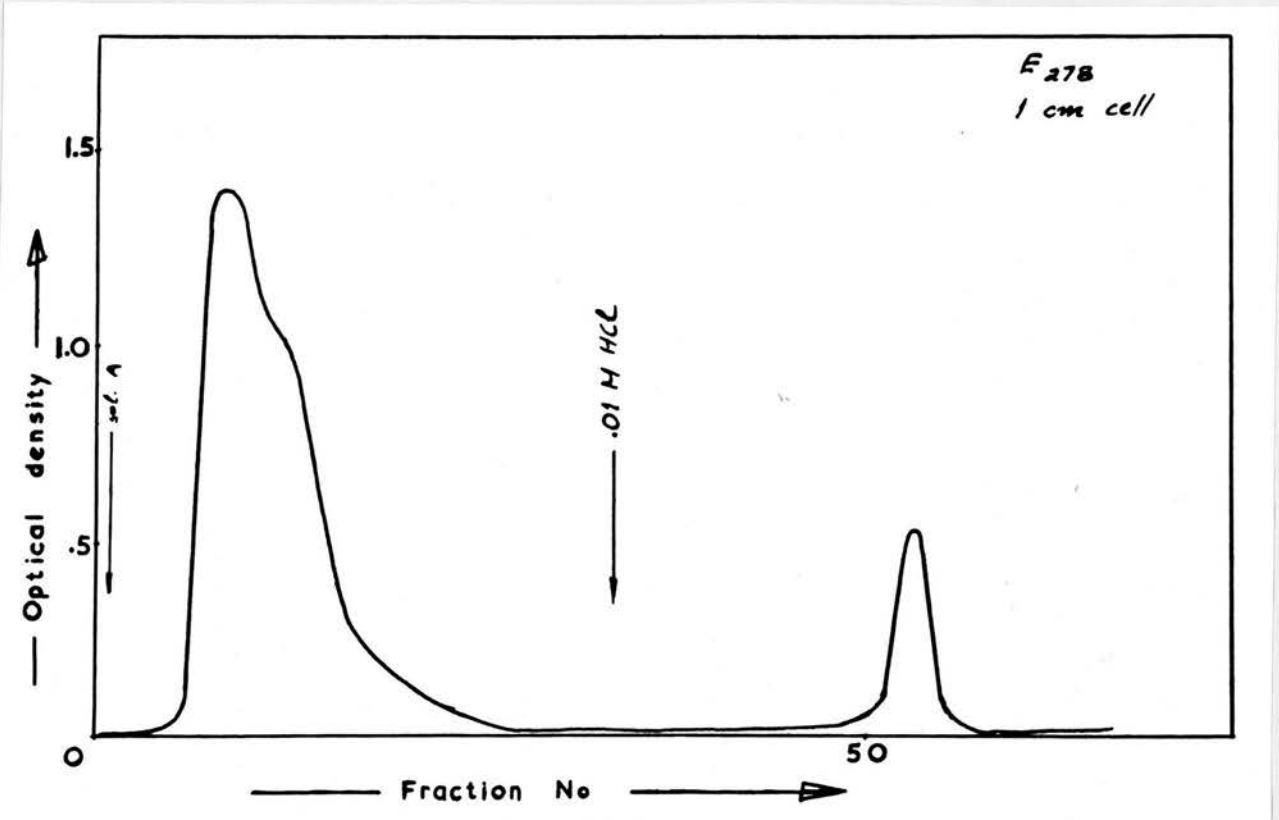


Fig. 31. CM-CELLULOSE CHROMATOGRAPHY OF a-HISTONE.

The fractions 50 - 55 were dialyzed against acetone. The yields were:

Fractions 5-35,400 mg. or 80% of starting material

Fractions 50 - 55,9 mg. or 1.8% of starting material.

The polyacrylamide gel electrophoresis of the above two fractions showed the major component to be a typical α-fraction. The minor one yielded very poor electrophoretic patterns and no conclusions could be drawn as to its composition.

FRACTIONATION OF RAT LIVER HISTONE ON CM-CELLULOSE CHROMATOGRAPHY

CM-cellulose chromatography was applied to the fractionation of rat liver histones with the addition of two further steps in the elution. The three steps as outlined above, namely acetate buffer containing salt, 0.01 N and 0.02 N hydrochloric acid, were found not to be sufficient for the elution of all the protein. The hydrochloric acid concentration had to be stepped up to 0.04 N and 0.2 N. These last two added two more peaks to the elution pattern. The rest of the technique was exactly as described in the first instance. The pattern obtained is shown on the graph in Fig. 32. The amount of protein applied to the column was 200 mg. The fractions taken together, and their yields are:

Fractions 5 - 50, 32 mg. or 16% of the starting material

Fractions 76-85, 3 mg. or 15% of the starting material

Fractions 102 - 113, 22 mg. or 11% of the starting material

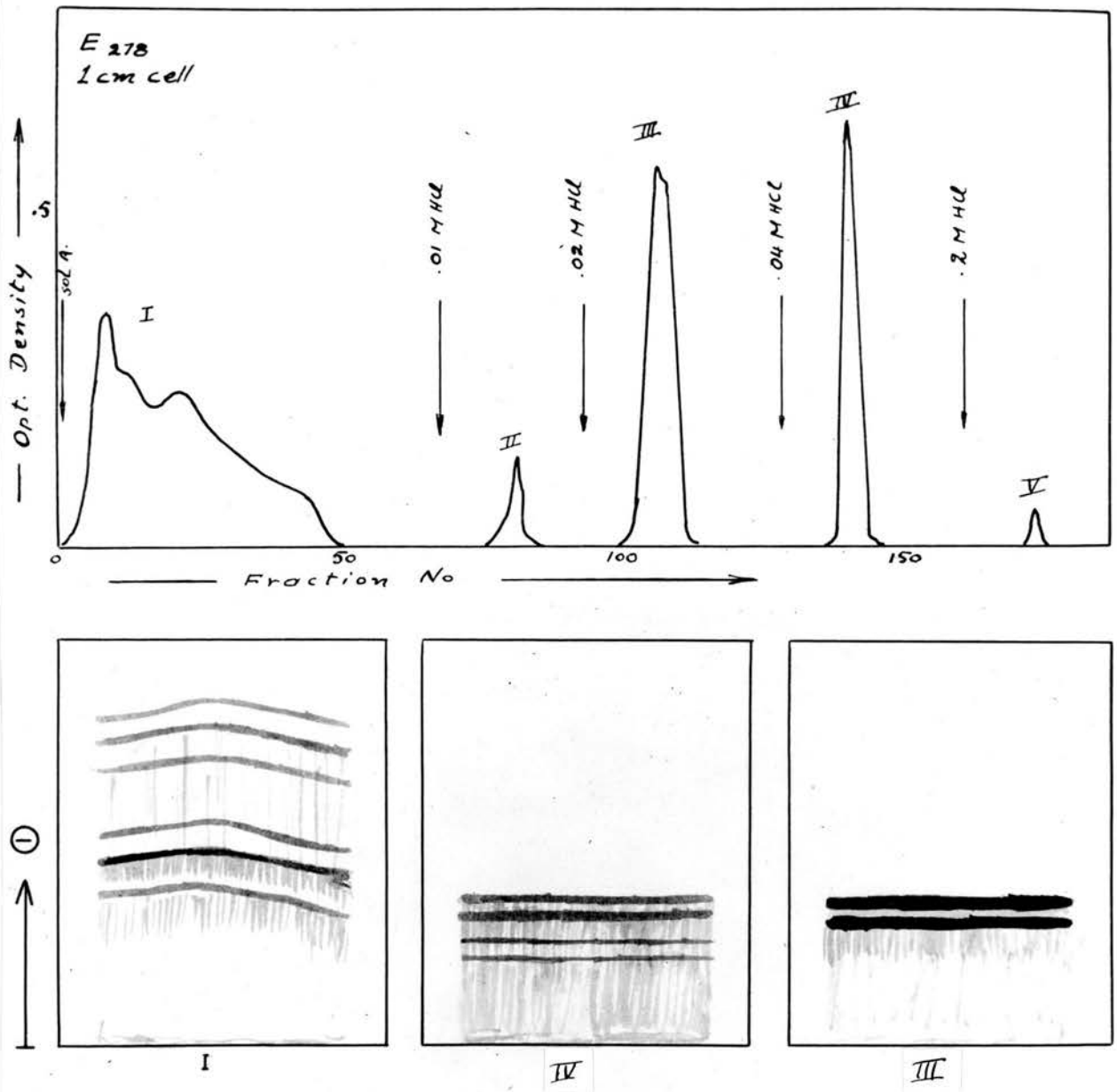


Fig. 32 CM CELLULOSE CHROMATOGRAPHY OF RAT LIVER HISTONE. ELECTROPHORETIC PATTERNS OF FRACTIONS I, III & IV.

I: GEL CONTAINING 75% ETHYLENE GLYCOL,

III & IV : LANTHANUM ACETATE GELS.

Fractions 135 - 147 11 mg. or 5.5% of the starting material

Fractions 172 - 176 ~1 mg. or 0.5% of the starting material

Overall yield 69 mg. or 34.5% of the starting material

The above fractions were identified in polyacrylamide gel electrophoresis as follows (Fig. 32).

Fractions 5 - 50 proved to be an α-histone preparation.

Fractions 76 - 85 gave no identifiable patterns although one band was clearly visible in the position usually occupied by the γ-histone bands. The fractions 102 - 113 gave a pattern attributable to a mixture of β and γ-histone and there was a lot of aggregated material in the starting line, which is typical of rat liver β-histone. The fractions 135 - 147 gave a β-histone pattern containing no detectable trace of γ-histone, and none of the turquoise α-bands. Finally the product precipitated from the fractions 172 - 176 could not be identified. In polyacrylamide gel electrophoresis it showed signs of heavy aggregation, as most of it did

not penetrate the gel, and the part that did showed no sharp bands at all.

Comment

The fractions obtained by CM-cellulose chromatography of rat liver histone, were somewhat less clearly defined than those obtained with calf thymus histone. The yield although very poor, was as expected, since this has always been the case with rat liver histones. Since the amount of rat liver histone was always limited, the CM-cellulose chromatography was found to be a better method than the ethanol precipitation technique, giving higher yields.

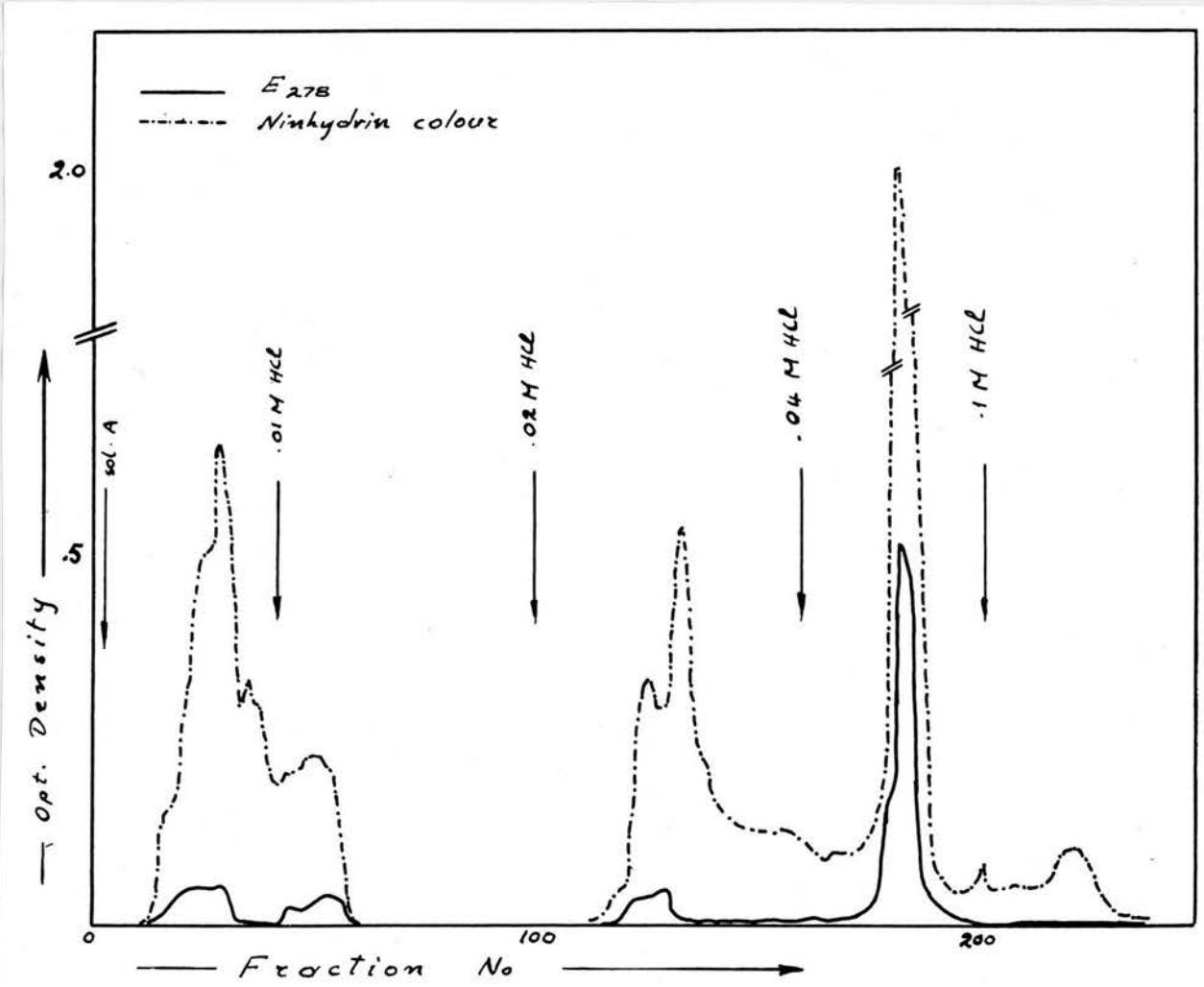


Fig. 33 CM-CELLULOSE CHROMATOGRAPHY OF REGENERATING RAT LIVER HISTONE.

FRACTIONATION OF REGENERATING RAT LIVER HISTONE ON CM-CELLULOSE CHROMATOGRAPHY

This fractionation was used only once and the results obtained are shown graphically in Fig. 33. While following the progress of the elution by measuring the optical density at 276 mμ. it was found necessary to perform a ninhydrin test in the fractions and obtain more clear cut results. The amount of histone used was 98 mg. and the recovery, corrected for samples withdrawn from each tube (5 ml. eluate) for ninhydrin estimation (0.5 ml.) and assay of radioactivity (0.4 ml.), was as follows:

Fractions 11 - 35, 3.16 mg. or 3.22% of the amount applied to the column.

Fractions 40 - 60, 1.5 mg. or 1.52% of the amount applied to the column.

Fractions 110 - 115, 6.1 mg. or 6.23% of the amount applied to the column.

Fractions 170 - 200, 22 mg. or 22.4% of the amount applied to the column.

Fractions 213 - 231 Traces

Overall yield 32.76 mg. or 33.34% of the amount applied to the column.

The overall yield of 33.34% is in good agreement with the 34.5% yield obtained with normal rat liver histones.

As the amount of the various fractions was very small and as the main aim of the experiment was to measure their radioactivity, no electrophoresis was run for identification purposes.

FRACTIONATION OF α-HISTONES WITH TRICHLOROACETIC ACID (TCA)

This method was originally used by Johns (1963) for the fractionation of the very lysine-rish histone fraction, extracted from calf thymus homogenate with 5% perchloric acid. A 50% solution of TCA was added to 12% (w/v) aqueous histone solution and the precipitates obtained were at a TCA concentration of 4.5% (0.28 M), at 11.5% (0.7 M) and at 17.5% (1.1 M). The yields reported were 9.1%, 67.8% and 4.6% respectively.

The method was performed with a 12% (w/v) solution of α-histones. Fifty per cent TCA solution was added slowly until the first traces of precipitate appeared, and the mixture was left at 2°C overnight. The precipitate formed was then removed by centrifugation. More TCA solution was added until a further precipitate just appeared and the mixture was again kept at 2°C overnight. In all subsequent preparations the amount that was found to produce a precipitate was added altogether and the formation of the precipitate was allowed to proceed overnight.

see page 186

TCA concentration °/o (w/v)	precipitate °/o	precipitate °/o (JOHNS 1963)
4.5	——	9.1 (x)
11.5	72.88	67.8 (a)
14.75	4.8	——
17.5	——	4.6 (b)
21	1.68	——
overall yield °/o	79.36	81.5

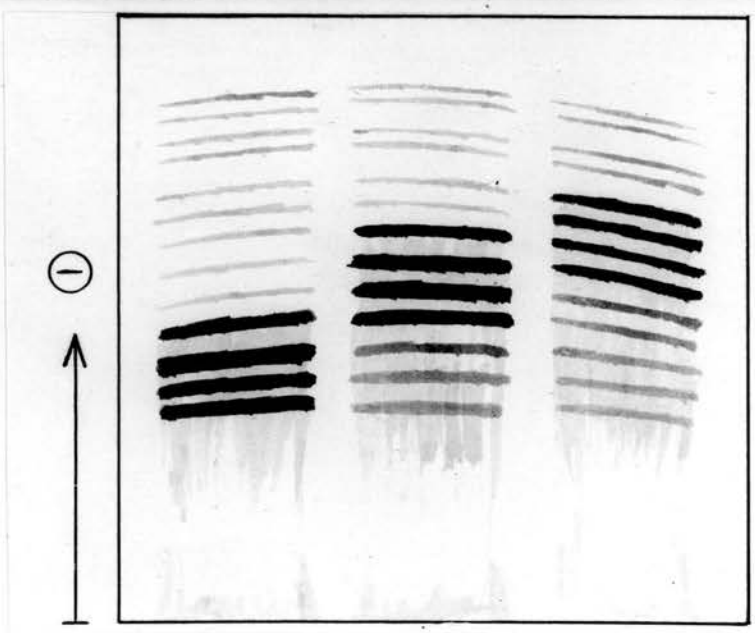


Fig. 34 PRECIPITATION OF a-HISTONES WITH TCA. POLYACRYLAMIDE GEL (ETHYLENE GLYCOL) ELECTROPHORESIS OF THE PRECIPITATES.

1. IN 11.5 °/o TCA. 2. IN 14.75 °/o TCA
3. IN 21 °/o TCA.

At 4.5% TCA there was no precipitate at all. The first appeared when the concentration of TCA reached 11.5%. The second precipitate came when the concentration of TCA reached 14.75% and the third at 21%. Further addition of TCA gave no precipitate. The yields of the first precipitate were between 68.5 and 77.25%; of the second between 2 and 7.5%, and of the third 1.6 to 1.7%. The overall yields were 77.7 to 81%.

All the precipitates formed were dissolved in 0.1 M hydrochloric acid, dialyzed against the same solvent and precipitated by dialysis against acetone. In the table shown in Fig. 34 the results obtained are compared with those reported by Johns. The differences are in the minor fractions as the major have in both cases precipitated at a TCA concentration of 11.5%. From electrophoresis on polyacrylamide gels it was noticed that the variation in the percentage yield of the precipitates was reflected by a difference in their electrophoretic patterns. One is shown in Fig. 34. It must be stressed that all the fractions were

mixtures enriched in some components, and in no case has a 'pure' subfraction, showing only a few of the bands of the pattern, been obtained. All the bands of the typical α-pattern were present in all the fractions, stained to a different extent.

The method unfortunately did not produce any useful results with the α-histones used in this laboratory. That the failure is due to a different starting material seems unlikely and the most likely explanation is that the methods used to test the purity of the sub-fractions (only amino acid analyses were mentioned) were not sufficiently fine.

USE OF ION EXCHANGE CHROMATOGRAPHY FOR THE FRACTIONATION OF THE MAJOR TCA PRECIPITATE OF THE α-HISTONE

Following the fractional precipitation of the α-histones, Johns (1963) attempted a further fractionation of the precipitate obtainable at 11.5% TCA concentration. This was performed by displacement chromatography on Amberlite IRC-50 cation exchange resin. According to the details reported, a column (7 x 2.5 cm.) containing 10 g. of IRC-50 resin in the hydrogen ion form was prepared. An experiment had shown that a column of this size was saturated by 100 mg. of the lysine-rich histone at pH 4, and yet it was hoped to fractionate a subfraction of TCA precipitation, containing twice this amount. From 200 mg. used, 50 mg. came through the column and 50 mg. were eluted from the column with 0.01 M hydrochloric acid.

This experiment was repeated three times. A column of the same dimensions (2.5 x 7 cm.) was prepared and packed with 10 g. of IRC-50 cation exchange resin. It was washed with 100 ml. of 1 M hydrochloric acid and water.

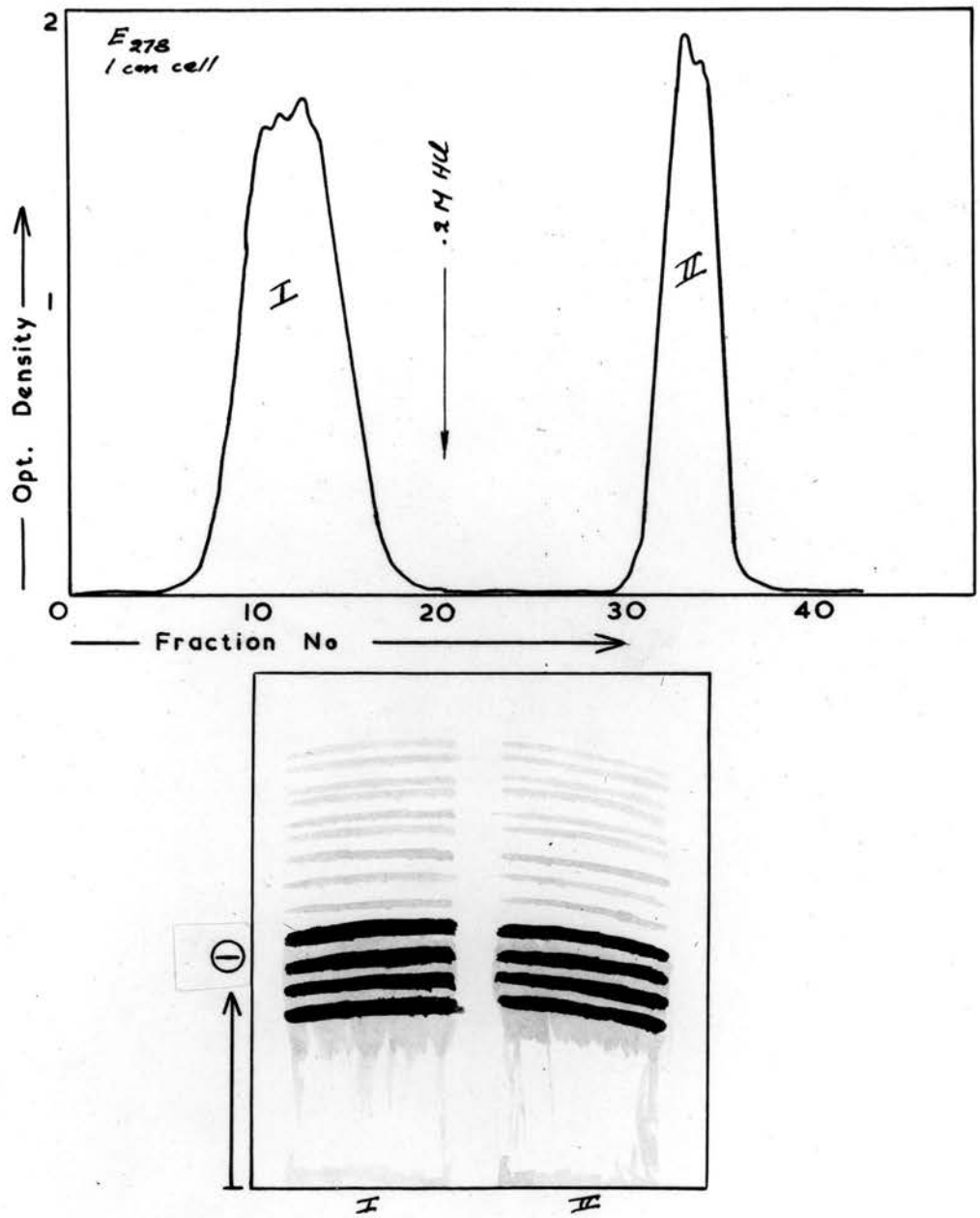


Fig. 35. AMBERLITE IRC-50 CHROMATOGRAPHY OF THE 11.5 % TCA PRECIPITATE FROM a-HISTONES, AND ELECTROPHORESIS OF THE TWO FRACTIONS IN ETHYLENE GLYCOL POLYACRYLAMIDE GEL.

The protein solution (200 mg. dissolved in 50 ml. of water, pH 4) was applied on the column at a rate of 1 ml./min., and fractions of 5 ml. were collected in an automatic fraction collector. When all the solution had been applied the column was developed with water and each fraction was tested for protein with ninhydrin. The first protein fraction appeared after 50 ml. of water had been added. The elution was then continued with 0.2 M hydrochloric acid, to elute the protein remaining on the column. One hundred millilitres of this new solution were used before there was no more protein coming out of the column (Fig. 35). The fractions 5 - 21 were mixed, and so were the fractions 27 - 38. The first batch containing fractions 5 - 21, was acidified with 5 ml. of 0.2 M hydrochloric acid, and dialyzed against acetone. Fractions 27 - 38 were dialyzed against acetone without any further acidification. The precipitates formed were collected by centrifugation, washed twice with acetone and twice with ether and were air dried. The yields were 46 mg. (23% of the starting material) for fractions 5 - 21, and 73 mg.

(36.5%) for fractions 27 to 38. The overall yield was 59.5%.

When electrophoresis was performed on the two samples, on the same gel containing ethylene glycol, they both showed the same number of bands, same intensities in staining, and had the same patterns generally (Fig. 35). Thus no separation occurred.

It was difficult from the beginning to visualize how separation would occur in this displacement chromatography system. Both fractions belong to the α-histone group and even to the same sub-fraction. The possibility therefore that they would demonstrate affinities for the resin which were different enough for a displacement to take place seemed remote.

Material fractionated	Fractions isolated	Yield* of each fraction	
		Ethanol chemical fractionation	CM-Cellulose chromatography
Calf thymus histone	α	21.4	15
	β	28.5	19
	γ	44.5	23
Rat liver histone	α	10.5	16
	β	23.9	5.5
	γ	16.2	11 **

Table 4A SUMMARY OF THE YIELDS OF HISTONE FRACTIONS OBTAINED BY ETHANOL CHEMICAL FRACTIONATION AND CM-CELLULOSE CHROMATOGRAPHY.

* Yield expressed as a percentage of the unfractionated starting material.

** Fraction containing β-histone.

Material Extracted		Yield as percentage (w/w) of material extracted	
		Perchloric acid extraction (extracts α-histone)	Ethanol-HCl extraction (extracts β-histone)
Calf Thymus	nuclei	4.2	7.1
	whole histone	12.5	24
	α-histone	82	
	β-histone	—	76
Rat liver whole histone		2	—

Table 4B SUMMARY OF THE YIELDS OF THE α AND β HISTONE FRACTIONS ISOLATED AFTER EXTRACTION WITH PERCHLORIC ACID AND ETHANOL-HYDROCHLORIC ACID MIXTURE.

Discussion

The comparison of the most common methods of extraction and fractionation of histones proved to be extremely interesting (Tables 4A and 4B). Particularly so was the application of techniques in which the original authors had used whole tissues, to the isolation of histone fractions from nuclei or crude unfractionated histone. The various workers have used different techniques and different criteria for the purity of their products. So the application of polyacrylamide gel electrophoresis permitted comparisons of the results that other methods yielded and allowed conclusions to be drawn about which would seem the method of choice.

The most commonly used criterion, amino acid analysis, was not considered to be sufficient and for this reason was not applied to test the purity of the fractions obtained. No fraction at all was electrophoretically pure, that is, showing a pattern of one band. Of course the possibility of a protein sample containing molecules of one species only, both as monomers and as polymers, and hence showing

more than one band on the pattern is not excluded. But in order to obtain more information about this aspect, each band would have to be eluted individually, enough material accumulated and then amino acid analysis performed. This was not attempted because of the enormous difficulties it presents. Firstly the exact location of each band on the gel would have to be found without staining, and secondly the proteins would have to be forced to migrate, most probably by electrophoresis, out of the gel into a solution. The amount of material on each band is so small, that the isolation of so many bands for analysis was considered impracticable. Nevertheless the purity achieved by the various methods although done on 'impure' fractions, gave good results.

The ethanol chemical fractionation, which was the method used in most cases, was found to give excellent reproducibility and, compared to the other methods, it gives the highest yields. It was successfully applied to the fractionation of histones of rat liver, and again in this case the yield of fractions was much higher

than any other method applied. The purity of the fractions obtained is considered very satisfactory. Occasionally small amounts of impurities were noticed in the various fractions but never to a high enough extent to influence the results.

The other method, also used for the fractionation of histone, was chromatography on CM-cellulose. This method, when applied to calf thymus histone, gave the same three fractions α, β, γ, whose patterns on polyacrylamide gel electrophoresis were almost identical to those obtained with fractions from the ethanol precipitation technique. Yet the yield of this method was always much lower than that of the chemical fractionation, except when small amounts (less than 100 mg.) of histone were used. When it was applied to the preparation of the rat liver histone fractions, the results were not as good. Of the five peaks, obtained on the elution diagram, only two were typical fractions; the first peak was the α-fraction and the fourth the β-fraction. A third peak was most probably the γ-fraction (in very small amount), another peak was clearly

a mixture of β and γ-fractions, and finally the last peak eluted with the more concentrated hydrochloric acid solution, could not be correlated with known patterns. The detectable differences in fraction purity being negligible, this method was used for rat liver histones, because these proteins were always available in very small amounts and the fractionation by chromatography gave better yields with these.

The extraction of the α-histones with perchloric acid was done with material that would not allow any possible contamination of histones with ribosomal basic proteins. The amount of α-histones obtained by chemical fractionation of whole histone was 21.4%. When extracted with 5% perchloric acid from whole histone the yield was 12.5% if histone-hydrochloride was used, and 16% in the case of histone-sulphate. From nuclei the amount extracted was 4.16% of their weight which is 16.5% of the amount of whole histone extractable from the nuclei. From these figures it would seem that not all the α-fraction is extracted with 5% perchloric acid. However the fraction purity is very high and certainly in polyacrylamide gel electrophoresis there is nothing 'missing'.

The remaining β and γ-fractions seem to be affected to some extent in the case of calf thymus histone, and denatured in histone isolated from rat liver. This conclusion was drawn from differences in the electrophoretic patterns, and in the case of rat liver from the insolubility in 0.1 M hydrochloric acid.

The extraction of β-histones with a mixture of ethanol and hydrochloric acid gave excellent results and extremely pure fractions. The patterns obtained in polyacrylamide gel electrophoresis were identical to the ones yielded by the same fraction isolated by the ethanol precipitation technique; further the fractions were cleaner with less aggregated material and trailing effects. The yields again were not as high as in the chemical fractionation, and the protein residue did not give clear patterns in polyacrylamide gel electrophoresis. This method was used several times when the need for β-histone was urgent, and proved very reliable. The β-fraction was extracted from whole histone.

The fractionation of α-histones by trichloroacetic acid precipitation did not give the expected results. This was the first method on the further fractionation of α-histone fraction tried, but unfortunately, there were no real sub-fractions. The three precipitates obtained in different TCA concentrations were found to be mixtures of the same components, enriched in some of them. The similarities would not probably be detected in starch gel electrophoresis, but were shown definitely in polyacrylamide gel. The differences in the amino acid analyses of the three precipitates given by Johns (1963) are easily explained, and may constitute evidence for the great number of proteins in the histones. If the numerous bands seen on electrophoresis, were just aggregates, then the amino acid analysis of the fractions should be the same. However, although all the bands occur in each fraction (Fig. 34), there is a difference in the amino acid composition of these fractions. In view of these facts this can be explained by the presence of different components in the bands.

The fractionation of one of the TCA precipitates reported by Johns (1963) could not be repeated, and it is difficult to see how it could have worked at all. The method of displacement chromatography applied on Amberlite IRC-50 cation exchange resin gave two fractions whose amino acid analyses are given in the Appendix. In these analyses there are some conflicting results. For example from a starting material containing no tyrosine, two fractions were obtained, one of which contained this amino acid. Also the amino acid composition of the fractions does not fit with that of the starting material, as in the case of proline; the unfractionated protein contains 6.5% and the fractions 9.6% and 8.6%. Differences also exist in the cases of glycine, leucine + isoleucine, lysine and arginine. The correspondence of the fractions with the ones obtained by Cruft et al.(1957b), also given in the Appendix, is very bad. One possible explanation of the differences observed in the chromatographic fractionation is the possibility of degradation of some of the components by the resin. The poor yield (50%) overall, favours this assumption.

The conclusions from the work of this section are the following:

1. for the fractionation of large amounts of histone the best method is ethanol precipitation.

2. for small amounts of histone, CM-cellulose gives better results.

3. use of fractional extraction techniques for α and β-histone should be made only if these fractions are needed urgently (in which case the residue should be discarded), or for further purification of fractions already isolated.

CHAPTER THREE

PART I

INCORPORATION OF ^{14}C-LABELLED AMINO ACIDS INTO THE HISTONES OF REGENERATING RAT LIVER

Introduction

The experiments aiming at the incorporation of ^{14}C-amino acids into histones were undertaken primarily to obtain these proteins in a labelled form, which might have been suitable for the autoradiography of polyacrylamide gels into which the labelled histones had migrated by electrophoresis. This would have given an indication of the rate of biosynthesis, not of whole histone-fractions, but of individual bands.

Since the early experiments of Rotherham, Irvin, Irvin, and Holbrook (1957), who reported a high rate of labelling of histones in a rat hepatoma, it became interesting to know the rate of biosynthesis of the different histone fractions. Investigations were undertaken by Holbrook, Evans and Irvin (1962), who used regenerating rat liver as their incorporating system, to determine the exact time of

biosynthesis of various cell components, during the cell life-cycle. They found differences in the rate of incorporation of Glycine-1-^{14}C into the various histone fractions (which they named I, II and III). However their fractionation of histones is unusual and their fractions cannot be correlated with those of other workers.

Bush and Steele (1964) studied the incorporation of labelled lysine into rat liver and hepatoma. There again, differences in the degree of incorporation into their four histone fractions were found, with the arginine-rich fraction showing the highest, and the lysine-rich one the lowest incorporation.

The system of Holbrook et al. (1962) was chosen for the incorporation experiments, because it was originally carried out with glycine, an amino acid contained in all histone fractions to about the same extent (6.7 to 9.7%) and also because it yielded the highest incorporation of about 105 counts/min./mg.protein/μC injected/100 g. rat body weight. In this system approximately one third of the liver

(the middle lobe) was removed and the regeneration resulted in a highly dividing non malignant tissue. The stress imposed on the animal is certainly less than that imposed by the removal of two thirds of the liver, which is a more commonly used method in partial hepatectomies.

Nineteen hours after the operation, a peak of incorporation of glycine-1-^{14}C into the histones was found by Holbrook et al. This result was taken into consideration in designing the following experiments.

Experimental

The removal of the middle lobe of the liver was performed on Wistar adult male rats weighing between 300 and 425 g. The animals were fed ad libidum pre- and post-operationally. Eighteen hours after the operation, the animals were injected intraperitoneally with glycine-1-^{14}C (specific activity 870 μC/mg.) or 'protein hydrolysate' (600 μC/mg.), dissolved in autoclaved distilled water. Both these products were obtained from 'The Radiochemical Centre', (Amersham). The protein hydrolysate had been prepared from disrupted cells of chlorella vulgaris, grown on $^{14}CO_2$ as the sole source of carbon. The analysis accompanying the product, determined chromatographically, was as follows:

L-alanine 5%, L-arginine 5%, L-aspartic acid 6%, glycine 3%, L-histidine 0 - 2%, L-leucine 9%, L-iso-leucine 4%, L-lysine 6%, L-phenylalanine 4%, L-proline 5%, L-serine 2%, L-threonine 2%, L-tyrosine 4% and L-valine 4%.

Total: 68 - 70%.

One hour after the injection, the animals were killed under anaesthesia by bleeding and the livers excised immediately. The nuclei were isolated according to the method described in the previous chapter and the histones were extracted with hydrochloric acid also as described. The yields of nuclei and histones are summarized in Table 3.

The radioactivity measurements were performed either with a Geiger-Müller counter, or in a liquid scintillation counter. The efficiencies of the countings were 7.5% and 80% respectively. The samples for counting in the Geiger-Müller counter were prepared by drying 1 ml. of protein solution containing about 1 mg./ml., to an infinite thickness layer on an aluminium planchette. The counting was usually done for 30 min., and the results expressed as disintegrations per minute (d.p.m.).

The counting in the liquid scintillation counter, was done by using an aqueous protein solution containing about 2 mg./ml; of this 0.4 ml. were taken and added to 5 ml. of NE220 liquid scintillator (Nuclear Enterprises) which

holds, up to 10% of its volume, water. Each sample, in duplicate, was counted for 10 min. in an automatic counter (Packard) and these results were also expressed as d.p.m.

Autoradiography

The preparation of the gels for autoradiography was carried out as follows. Polyacrylamide gel electrophoresis was performed either in lanthanum-acetate or in ethylene glycol gels. The gels were stained with Amido Black, and dried with ethanol as already described. The dry gels were left in a mechanical press for one day to flatten them completely, glued on a piece of glass with 'Araldite' and again placed in the press for a further two days. They were then polished with fine sand paper, very fine emery paper and finally with 'diamantine powder'. The surface obtained was absolutely flat and highly glossy.

The films used for the autoradiographies were 'No Screen Ilford X-ray'. They were placed with the emulsion facing the gel and kept in position with another piece of glass on top of them. The two glass plates were held

together with 'sellotape'. The whole was put in a light-proof envelope and left in a calcium chloride desiccator during the period of exposure. The use of the desiccator was dictated by the fact that the gels absorb humidity from the atmosphere, and swell unevenly. The films were developed in a high contrast developer.

Calculation of exposure time

As a basis for the calculation of exposure time needed, the data given by Francis, Mulligan and Wormal (1959) was taken. According to these authors a minimum of 100 electrons/min./ cm^2 of the film area is needed to produce a clear print after one week's exposure. However, the specific activity of the protein used for autoradiography was 3220 d.p.m./mg. and its concentration in the samples prepared for electrophoresis was 4 mg./ml. (12800 d.p.m./ml.). About 0.05 ml. were taken for an electrophoretic run thus giving 12,800 x 0.05 = 640 d.p.m. If these were spread over an area of 20 cm^2 the density would be 640 / 20 = 32 d.p.m./cm^2.

This figure should be again reduced by approximately 70%, due to the geometry of the system and absorption, which left an expected 10 electrons to reach one cm^2 of the film every minute. This should give, according to the above authors, a clear autoradiography in ten weeks. If the fact that the proteins are accumulated in bands and not spread all over the area is taken into account, then this figure should be considerably shorter and the correct length of time could be found by trials.

Rat weight g	Liver * weight g	µC / 100g body weight		Histone extracted mg	dpm / mg ** of histone
		Glycine	Protein Hydrolysate		
390	9.85	12.8	——	14	1900
360	9.1	14	——	13	1910
300	7.3	16.5	——	10.9	1970
300	7.4	16	——	10.2	2050
425	10.13	23.5	——	14.7	3220
420	11	——	23.8	15	1730
405	10.1	——	24.6	17	1800

Table 5. INCORPORATION OF GLYCINE-1-^{14}C AND PROTEIN HYDROLYSATE-^{14}C INTO THE HISTONES OF REGENERATING RAT LIVER.

* extracted 19 hours after partial hepatectomy.

** dpm denotes disintegrations per minute.

Results

The incorporation experiments were done with increasing doses from 50 μc. up to 100 μc. per rat in order to find the dose that would give the greatest incorporation. The results are summarized in Table 5. From this Table it can be immediately seen that the highest incorporation into histones was obtained with Glycine-1-^{14}C, injected at a dose of 23.5 μC/ 100 g. body weight. This was, in fact, the highest dose injected. The administration of protein hydrolysate up to the same dose gave less incorporation into the histones.

Note: During the procedure of the extraction of nuclei and histones the radioactivity was checked continuously. From the total amount of d.p.m. of the supernatant of histone extraction, and the total amount of d.p.m. given by the histones after precipitation, it was found that much of the activity was missing. This 'missing activity' was found in the acetone bath that had been used for the precipitation of histones by dialysis. This finding was further investigated and the results are given in the second part of this chapter.

Autoradiography

The autoradiography has not yielded the results expected. The exposure times have been up to five months and the most that could be seen on the film (after five month's exposure), was a sharp blackening on the starting line, a haze over the whole migration area and a suspicion of a band in the position where usually the γ-histone migrates. Nothing else could be seen on the film and even that mentioned above needed very careful observation in order to be distinguished.

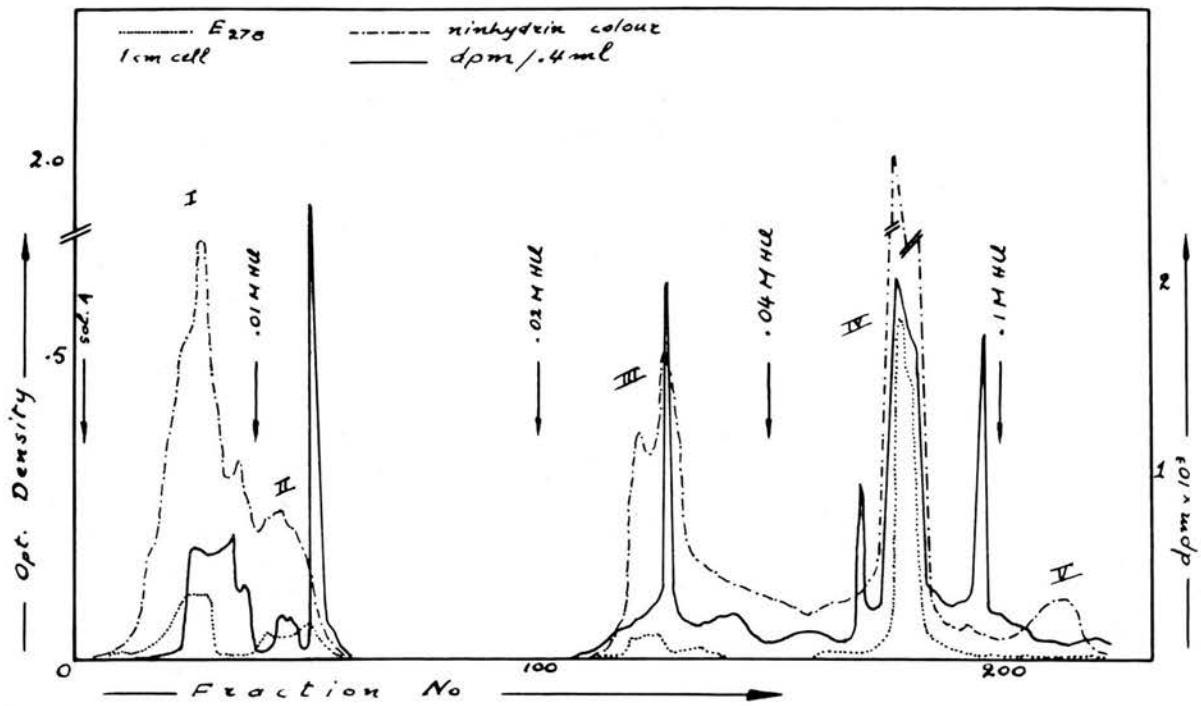
This failure is most probably due to the low specific activity of the labelled histones, and absorption of the β-particles by the dried gel. Only the disintegration of molecules, on, or very near, the surface of the gel could have caused any spots on the film, and the number of these molecules was certainly too small to give any useful results.

FRACTIONATION OF THE REGENERATING RAT LIVER ^{14}C-HISTONES

When the results of the autoradiography were found to be negative, all the existing amount of labelled and non-labelled histone from regenerating rat liver was fractionated on a CM-cellulose column, in order to separate the three main fractions and count their activities individually.

The material available was 98 mg. and showed 985 d.p.m./mg. The technique used was the one described in Chapter Two. The elution of the column was followed by the ultraviolet absorption at 276 mμ., by the ninhydrin colour of the fractions, and by measuring the radioactivity in every fraction separately. The results are shown in Fig. 36.

An unusual peak (peak II) was found in the region of the α-histones and this was tested separately from the rest of the α-fraction (peak I). There was also a peak (peak V) shown only by ninhydrin, but this contained no radioactive material at all. Because the amount of the fractions eluted was extremely small it was impossible to perform electrophoretic analysis.



Peak	Amount of histone recovered mg	Amount of histone counted mg	Specific activity dpm / mg	Comparative specific activity *
I	2.6	1.310	1772	2.97
II	1.2	.416	596	1
III	5	1.180	790	1.33
IV	18	1.080	1030	1.73
V	1.5	1.006	—	—

Fig. 36 FRACTIONATION OF REGENERATING RAT LIVER HISTONE BY CM-CELLULOSE CHROMATOGRAPHY.

* Specific activity compared to the specific activity of peak II histone, taken as unity.

Discussion

The incorporation experiments with regenerating rat liver and glycine-1-^{14}C initiated by Holbrook et al. (1962) yielded a histone having approximately 105 d.p.m./mg. protein/μC injected/100 g. body weight. The authors did not specify whether or not their countings were corrected for the efficiency of their equipment. For the sake of comparison it was presumed that they were.

When the same experiments were performed with greater doses of glycine-1-^{14}C (23.5 μC/100 g. body weight, instead of 5.4 to 6) the incorporation was found to be 30% greater (137 d.p.m./mg. protein/μC injected/100 g. body weight). Moreover, the specific activity (d.p.m./mg. protein) was four times higher (3200 instead of 800), but unfortunately not high enough to give results by autoradiography.

The results of CM-cellulose chromatography (Fig. 36) showed the α-histones to be fractionated into two sub-fractions (peaks I and II), with widely differing incorporation rates. Peak I had the highest incorporation rate of all the protein fractions, while peak II the lowest.

Peak III was expected to be a mixture of β and γ-histones, while Peak IV the β-fraction.

The fractionation of histones labelled in the same way by Holbrook et al. (1962), was performed by extracting disrupted nuclei with 0.05 M sodium citrate solution (Fraction I) followed by an extraction with 1 M solution of sodium chloride which yielded a soluble fraction (Fraction II) and an insoluble one (Fraction III). Fraction II was reported earlier by the same workers (Holbrook, Irvin, Irvin and Rotherham, 1960) to contain very lysine-rich and slightly lysine-rich histones (α and γ respectively). Therefore the comparison of the results reported in this thesis, with those of the above workers is impracticable.

The results reported by Bush et al. (1964) showed that in 'normal' rat liver the rate of uptake of lysine-^{14}C into histone fractions (corrected for lysine content of each fraction), was lowest in the very lysine-rich histones, and highest in the arginine-rich. The uptake of the same amino acid by the histone fractions of rat hepatoma was roughly the same. This is

exactly the opposite of what has been found to be the case with regenerating rat liver. The fact that the differences between normal rat liver and hepatoma are non-existent, makes it seem unlikely that the regenerating rat liver should show the opposite effect. The only likely explanation is that the differences are due to the different amino acids used for the uptake experiments.

PART II

NINHYDRIN-POSITIVE MATERIAL DIFFUSING THROUGH THE DIALYSIS BAG

Introduction

The presence of radioactive ninhydrin-positive material diffusing out of the dialysis tubing during the precipitation of histones, was detected, as has been already mentioned, from the difference in radioactivity counting between the supernatants of the histone extraction, and the histones after precipitation. The difference was of the order of 45% and it was beyond the limits of experimental error.

There have already been reports in the literature that the histones migrate through dialysis tubing. Bakay, Kirschner and Toennies (1957), Luck et al. (1958), and finally Smillie, Marko and Butler (1955) reported that the histones diffuse through the dialysis sac at pH's below 2.6. At other pH's, such diffusion was reported by Ui (1957a) and Crompton et al. (1957).

It was therefore suspected that some of the histones, most probably of the low molecular weight α-group, may diffuse into the dialysis bath and are consequently lost. The following investigations were undertaken to find out more about the nature of this diffusate.

Experimental

Ninhydrin quantitative amino acid estimation

The hydrindantin-ninhydrin quantitative technique of Moore and Stein (1954) was applied whenever mentioned. A reference solution of 0.5 μM/ml. glycine was used throughout.

Ninhydrin spraying solution

A solution of 0.5% ninhydrin in 95% acetone and 5% water was used throughout, and the chromatograms allowed to develop at room temperature for 24 hr.

Hydrolysis

The hydrolysis of the samples was performed in sealed 'pyrex' glass tubes, with 6 N HCl at 110°C for 24 hr. as described by Cramer (1954). The hydrochloric acid was evaporated *in vacuo* by heating at 80°C and continually shaking. The dry product was dissolved in water, which was re-evaporated under the same conditions in order to remove any remaining traces of hydrochloric acid.

Desalting

A modification of the technique of Boulanger and Biserte (1951) was used. These authors used Permutit 50 ion exchange resin for desalting plasma samples. Instead of Permutit, Dowex 50 was found to work satisfactorily, and the salt was eliminated to the extent of 99.99%.

The column was packed with the Dowex 50, and then the resin was regenerated by passing a large volume of 2 N hydrochloric acid through the column. After washing with distilled water the column was then ready for use. The sample was applied in 1% acetic acid solution, at a flow rate of 0.5 ml./min. The column was washed with 500 ml. of water, and then the elution was carried out with 1 N ammonia solution at the same flow rate. According to Boulanger et al. (1951) the elution of amino acids and peptides was over when 100 ml. after the appearance of ammonia in the eluate were collected. This was found to be the case.

Concentration of the solutions

All solutions (acetone from dialysis bath and the fractions of the desalting column eluate)

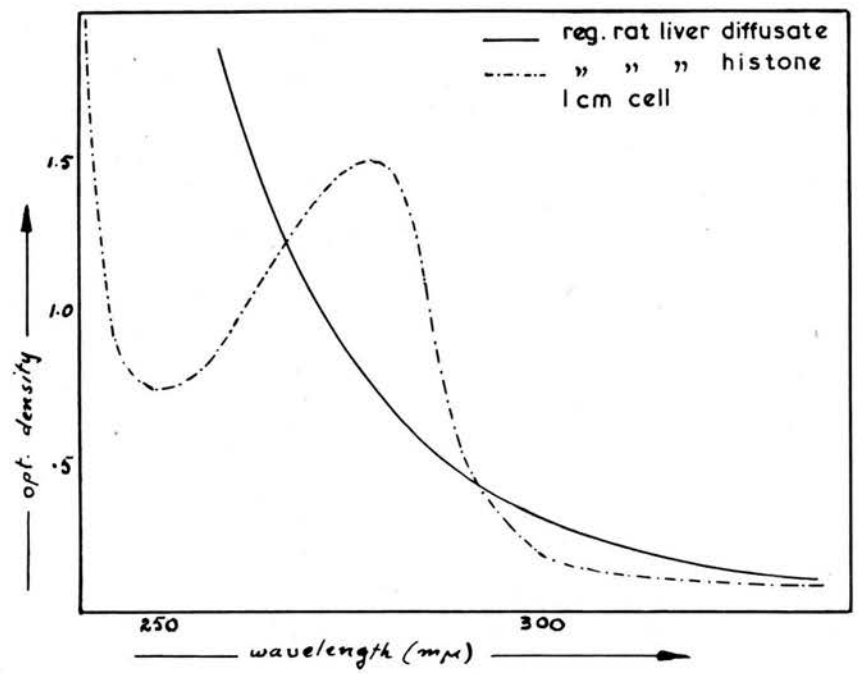
were concentrated in a rotary evaporator under tap vacuum and mild heating (35 - 40°C).

Paper chromatography

For both ascending and descending paper chromatography the solvent used was n-Butanol-water-acetic acid (63:27:10). Either Whatman No. 1 or No. 3 papers were used.

Scanning of chromatogram for radioactivity

Chromatograms were scanned for radioactivity in a BTL 'Radioactive chromatogram scanner' which utilizes an argon gas-flow counter. Using this instrument 1 cm. x 2.5 cm. sections of the chromatogram were counted simultaneously for 30 min.



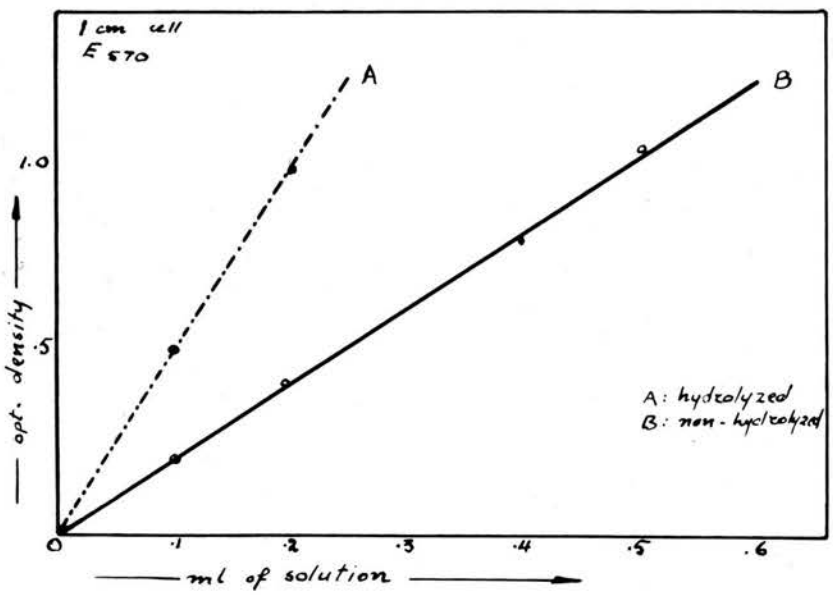


Fig. 37 ULTRAVIOLET ABSORPTION CURVE OF THE REGENERATING RAT LIVER DIFFUSATE, AND ESTIMATION OF THE NINHYDRIN POSITIVE MATERIAL BEFORE AND AFTER HYDROLYSIS.

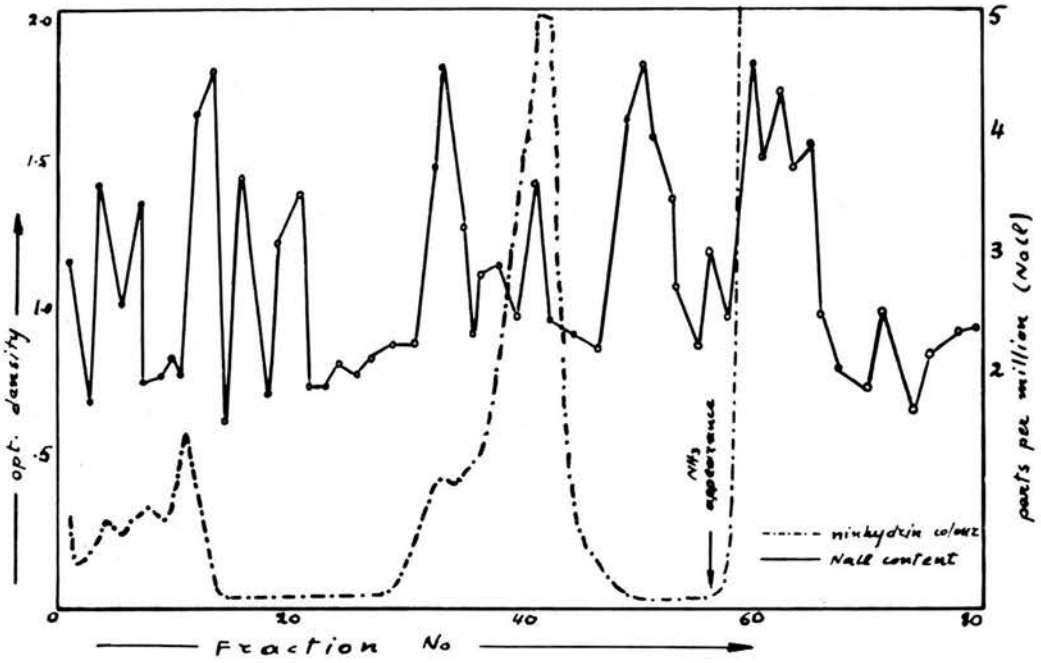
The UV absorption spectrum of regenerating rat liver histone obtained with a 0·3% (w/v) solution in 0·1 N HCl is included for comparison.

Results

After the labelled histones had been extracted from the isolated nuclei of regenerating rat liver, the mixed supernatants of the extractions (v = 150 ml.) counted 87,400 d.p.m. Yet the histones isolated had a total of only 47,360 d.p.m. The difference of 40,000 d.p.m. was found to be due to material diffusing into the acetone bath (used to precipitate histones by dialysis). The acetone bath was neutralized with sodium hydroxide and evaporated to dryness in the rotary evaporator. The dry material (which is referred to as diffusate) was collected with 10 ml. of distilled water.

The ultraviolet absorption spectrum of this material had little resemblance to that of regenerating rat liver histone (Fig. 37).

The solution was then tested with hydrindantin-ninhydrin solution, and the colour obtained was found to be equivalent to that given by 1.15 μM/ml. glycine (Fig. 37). The possibility that the ninhydrin colour was due simply to the glycine injected was excluded because the amount injected (0.11 mg.) was very small, compared to the material isolated (2.59 mg).



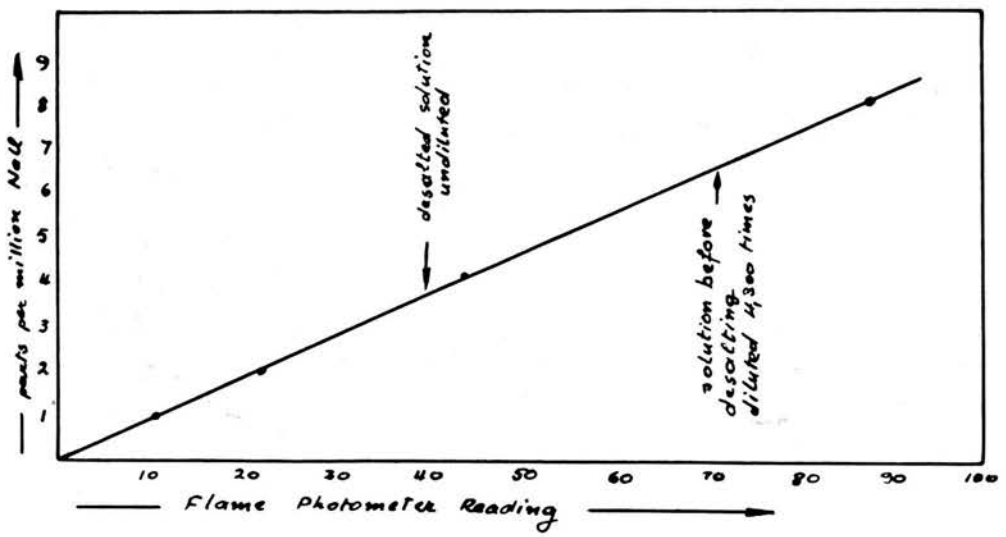


Fig. 38 DESALTING OF THE REGENERATING RAT LIVER DIFFUSATE BY DOWEX-50 RESIN, AND CALIBRATION CURVE OF THE FLAME PHOTOMETER.

A 1 ml. sample was hydrolysed with hydrochloric acid, and after evaporation of the excess acid the dry product was redissolved in 1 ml. water. A ninhydrin-hydrindantin estimation showed an increase of 2.4 times in the intensity of ninhydrin colour (Fig. 37).

The solution of the diffusate was then desalted using a column (16 mm x 20 cm) of Dowex 50 H^+ as already described. The elution was followed by testing the fractions for ninhydrin-positive material, and sodium chloride content. In fraction 57, ammonia appeared (pH of the eluate 9) and the ninhydrin estimation was stopped (Fig. 38). The elution was continued until 80 fractions (of 5 ml. each) were collected. These were mixed together and the eluate was evaporated in the rotary evaporator. The dry material was dissolved in 5 ml. of distilled water (its initial volume) and the salt content measured in the flame photometer (Fig. 38). The content was down from 2.8% to 3.6 parts per million (0.001% of the original salt left).

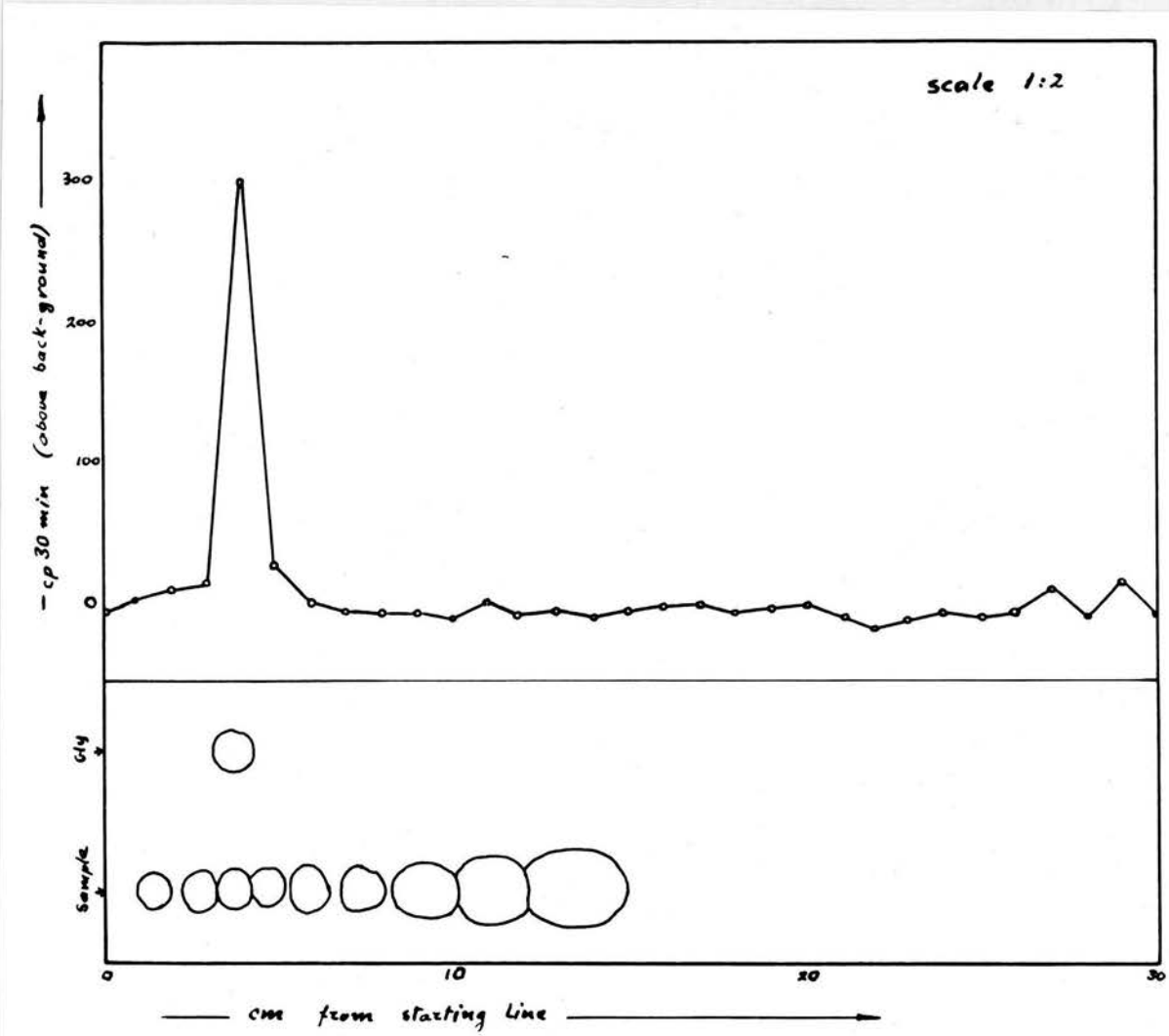


Fig. 39 ASCENDING CHROMATOGRAPHY OF THE REGENERATING RAT LIVER DIFFUSATE, AND SCANNING OF THE CHROMATOGRAM.

This desalted solution was then used for ascending chromatographic analysis; 0.2 ml. were put to run on a Whatman No. 3 paper, along with a glycine standard (0.01 ml. of a 3 mg./ml. solution). The duration of the run was 16 hr. and after developing the chromatogram with ninhydrin spray, it was scanned for radioactivity. The results are shown in Fig. 39 where it is clear that there is only one peak of radioactivity corresponding to glycine.

Following these preliminary investigations, and as there was no more material available, it was decided to investigate the case in a calf thymus preparation.

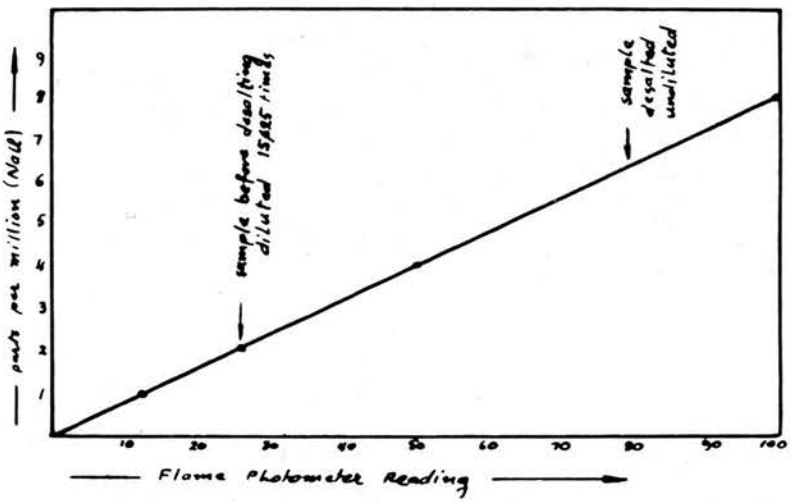


Fig. 40 ESTIMATION OF NaCl CONTENT OF THE CALF THYMUS DIFFUSATE.

DIFFUSATE FROM CALF THYMUS NUCLEI

Nuclei (11 g.) from calf thymus, were extracted three times with 100 ml. 0.1 N hydrochloric acid each time. The three combined supernatants were dialyzed against four litres 'Analar' acetone for two days. The mixture of acetone-hydrochloric acid from outside the dialysis sac was then neutralized with sodium hydroxide, and evaporated to dryness in the rotary evaporator. The precipitate was dissolved in 250 ml. of 1% acetic acid and applied to a column (4 x 50 cm.) of Dowex 50. The eluate containing the ninhydrin-positive material was evaporated to dryness in the rotary evaporator and redissolved in 50 ml. of distilled water. This desalting procedure was checked using the flame photometer (Fig. 40). (Note: the overall amount of ninhydrin colouration was estimated before and after passing the solution through the desalting column, in order to find out if anything is lost, and it was found that the recovery was quantitative).

The ninhydrin colour of the diffusate was estimated before and after hydrolysis with 6 N hydrochloric acid. The results showed that the

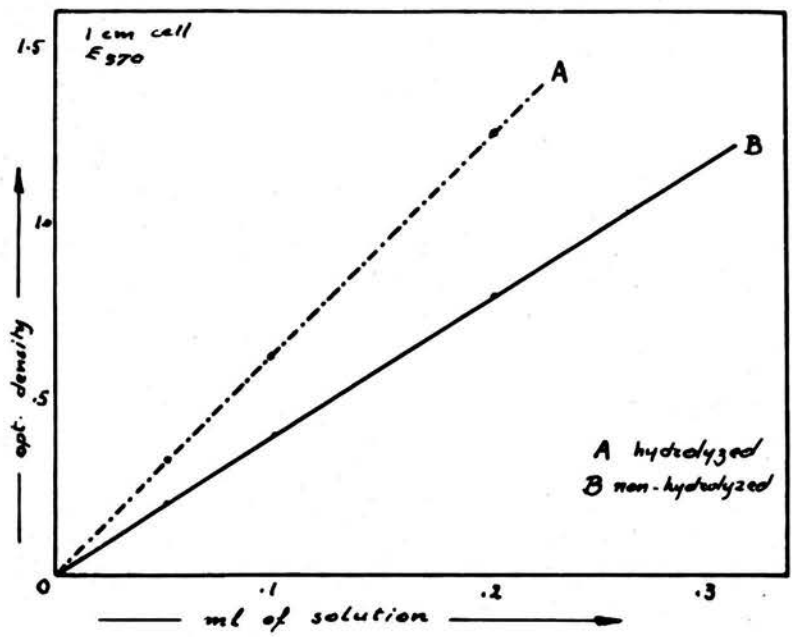
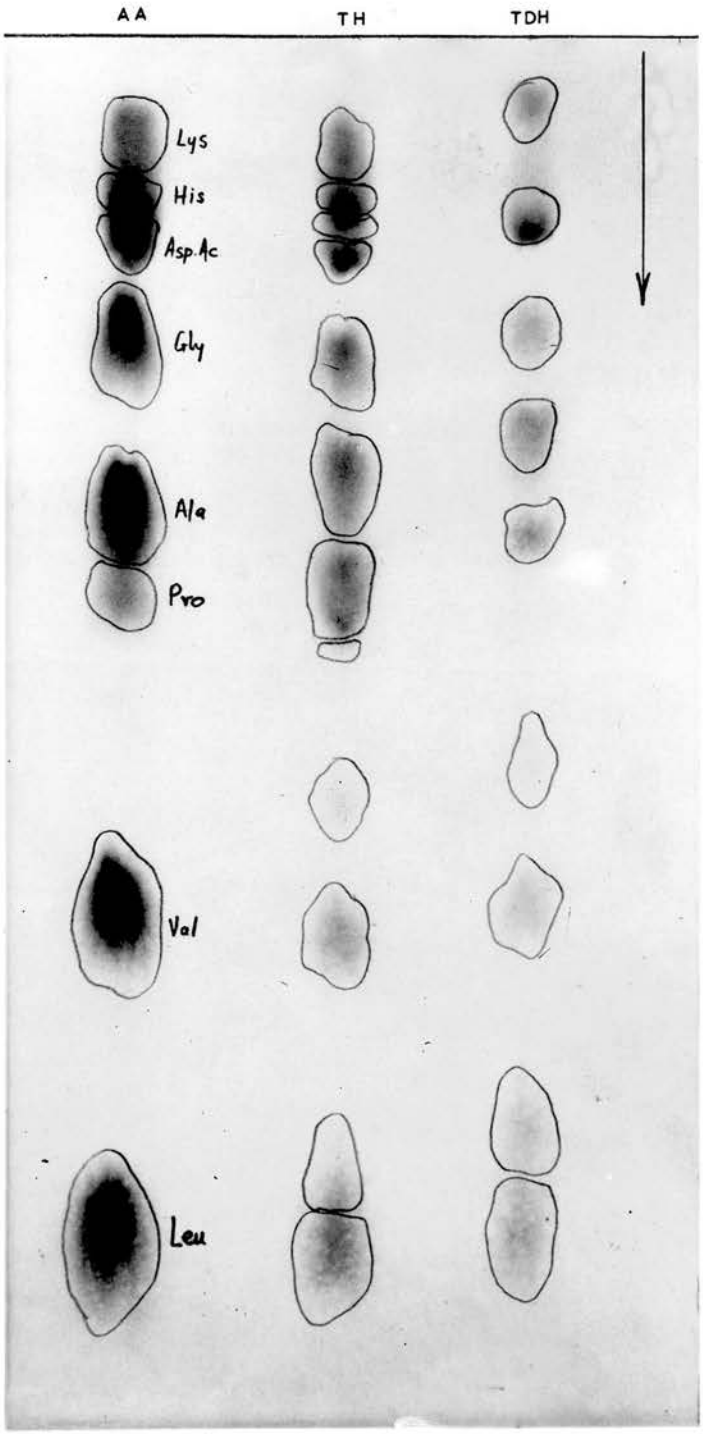


Fig. 41 NINHYDRIN COLOUR BEFORE AND AFTER HYDROLYSIS OF THE CALF THYMUS DIFFUSATE.

Fig. 42 DESCENDING CHROMATOGRAPHY OF HYDROLYZED CALF THYMUS DIFFUSATE TOGETHER WITH HYDROLYZED CALF THYMUS HISTONE AND STANDARD AMINO ACID MIXTURE.



ninhydrin colour had increased 1.7 times after the hydrolysis (Fig. 41).

Descending chromatography (Whatman No. 1 paper, 16 hr.) was performed with three samples simultaneously (Fig. 42). The first sample (marked TDH) was the hydrolyzed diffusate from calf thymus nuclei. The sample shown as TH was hydrolyzed calf thymus whole histone, and the sample AA, a standard mixture of amino acids containing: lysine 6 mg./ml., histidine 6 mg./ml., glycine 3 mg./ml., aspartic acid 5 mg./ml., proline 5 mg./ml., alanine 4 mg./ml., leucine 5 mg./ml. and valine 5 mg./ml. 0.01 ml. of this amino acid mixture and 0.2 ml. of the other two samples were used.

The differences between the patterns of the various samples after spraying with ninhydrin show that hydrolyzed calf thymus histone has more spots than the diffusate from the same tissue.

Discussion

From the evidence presented and especially from the intensity of the ninhydrin colour before and after hydrolysis, it can safely be concluded that the diffusate consists of amino acids and some peptides.

The diffusate might have originated from one of the following sources. 1) From histones or other proteins during extraction with hydrochloric acid. 2) From the nucleus where it may have existed as such or, 3) from the nucleus but bound to soluble ribonucleic acid (S-RNA).

1. The possibility that histone is partly hydrolyzed, or a histone fraction completely destroyed does not seem remote. However, the chromatogram shown in Fig. 41 of the hydrolyzed calf thymus whole histone (TH), has more spots than the hydrolyzed diffusate (TDH), and in all chromatograms the patterns have shown the same differences. It seems more probable that if a hydrolysis occurs, then it is of either a fraction consisting of a few per cent of whole histone, or of another protein of the nucleus.

2. If the assumption is made that the amino acids and small peptides exist as such in the nucleus, the question arises why they did not wash out during the preparation of the nuclei. It is recalled that during their isolation, the nuclei are washed about eight times with 1% acetic acid. The material diffusing out of the dialysis tubing, is certainly soluble in this solvent.

3. The third possible explanation is that these amino acids and peptides are in the nucleus, not free, but bound to S-RNA in the process of being incorporated into protein molecules. This would explain why they did not wash out during the purification of nuclei with 1% acetic acid, but they did when hydrochloric acid was used.

The only question that can be answered with certainty is that the diffusate is not normal histone.

GENERAL DISCUSSION

The electrophoretic analysis of histones in polyacrylamide gels, prepared in aqueous and organic solvent mixtures, demonstrated that the complexity of this protein group is possibly greater than was previously thought. It also helped in evaluating the most commonly applied fractionation techniques. The general finding was that a 'pure' histone fraction consisting of a single molecular species has yet to be obtained. The numerous new sub-fractions revealed are most probably entities in themselves and not artifacts of the various methods. Whether or not these sub-fractions contain molecules of one species only, remains to be proved by further analysis.

For the time being it can be considered as evidence for Stedman's hypothesis, that histones play a role in the expression of genetic information by blocking parts of the DNA and allowing only the remaining portions to function.

If the role of histones in the cell economy was simply the neutralization of the acid groups on the DNA molecules, or the stabilization and protection of DNA itself

(Vendrely et al. 1960), then there is no simple explanation of why so many species of histones should exist in the nuclei of one tissue.

The great number of histones (thirty or possibly more) can account for the cell specificity of this protein group, if this is finally proved to be the case. The combination of some or possibly all of the histones can theoretically provide a system of infinite variations and hence infinite specific functions. Again in order to know more about this, the analysis should be more complete and extended to as many different species and variety of tissues as possible.

The experiments with radioisotopes, performed in order to find out more about the individual fractions by autoradiography (in this case to establish a technique for the measurement of the uptake of amino acids by the various sub-fractions), did not yield the results expected. The low rate of uptake of amino acids by these proteins prevented the autoradiographic method from being successful. Different incorporation systems should be designed in order to attempt the production of

histones with higher specific activity. Liver perfusion, possibly by perfusing a regenerating rat liver, may provide the product with the desired higher specific activity. The 'closed' system, under which perfusion experiments are performed, would prevent the dilution of the labelled amino acid and could possibly provide the answer to the problem.

The rate of uptake of labelled glycine by the different histone fractions of regenerating rat liver was found to be different for each fraction. The significance of this result cannot be fully understood, because the role of histones is not known. They are known to suppress DNA-dependent RNA synthesis, but the extent to which each histone fraction is responsible for this, is far from being generally accepted. Various workers have published contradictory results. Huang, Bonner and Murray (1964), and Barr and Butler (1962) found the lysine-rich histones to be the most actively suppressing fraction, but Allfrey, Littau and Mirsky (1963) and Hindley (1963) found the arginine-rich histones to show the

greatest suppression activity. Moreover, even the uptake of amino acids by the various fractions was found to be the opposite from what has been published (Bush et al. 1964).

This confliction in the results reported is probably due to the different techniques and criteria applied. Unless the analysis of histones is completed the contradictory results will persist, and the complexity and role(s) of this protein group will remain an unanswered question.

ACKNOWLEDGEMENTS

I wish to thank Professor R.B. Fisher, in whose Department these investigations were carried out, and to express my gratitude to Dr. H.J. Cruft for his guidance and helpful criticism.

I also wish to express my indebtedness to the Greek Atomic Energy Commission without whose financial support this study would have been impossible.

APPENDICES

The Nomenclature of Histone Fractions*

Lysine/arginine molar ratio	Nomenclature				
Above 4	α	A	I	f1	E2
Between 1 and 4	β	B	II	f2	E1
Less than 1	γ	-	III-IV	f3	E3
Reference	1	2	3	4	5

* Taken from Phillips (1962).

References: 1. Cruft et al. (1954, 1957a)
2. Crampton et al. (1955)
3. Luck et al. (1958)
4. Johns et al. (1960)
5. Johns et al. (1961)

Amino Acid Analyses of the Lysine-rich (α) Histones

Values as moles per 100 moles of all amino acids.
Taken from Phillips (1962)

Amino Acid	Calf Thymus			Rat Liver
	α_1	α_2	α_3	
Aspartic Acid	0.3	2.4	4.4	5.0
Glutamic Acid	0.6	3.9	5.1	
Glycine	3.7	9.0	10.6	7.5
Alanine	31.6	22.8	18.4	23.4
Valine	3.1	4.7	5.9	4.4
Leucine	0.6	4.4	7.5	5.7
Isoleucine	0	1.0	2.0	
Phenylalanine	0	0.5	1.2	0.4
Tyrosine	0	0.6	1.7	0.55
Serine	2.6	5.0	6.9	5.7
Threonine	4.3	5.2	4.4	5.3
Proline	14.5	9.8	6.6	10.4
Methionine	0	0	0.5	0.4
Arginine	0	4.2	4.4	2
Histidine	0	0.3	1.2	0.1
Lysine	38.7	26.2	19.2	29.1
Reference	1	1	1	2

1. Cruft *et al.* (1957b)
2. Holbrook *et al.* (1960)

Amino Acid Analysis of the Arginine-rich (β) Histones

Values as moles per 100 moles of amino acids (calf thymus)

As N per cent of recovered total nitrogen (rat liver)

Amino Acid	Calf Thymus	Rat Liver
Aspartic Acid	5.8	8.8
Glutamic Acid	9.7	
Glycine	8.5	6.4
Alanine	11.6	8.3
Valine	5.9	4.2
Leucine	10.1	9.3
Isoleucine	5.0	
Phenylalanine	2.0	1.0
Tyrosine	2.8	1.7
Serine	3.7	3.0
Threonine	5.9	4.4
Proline	3.7	2.3
Methionine	1.3	0.8
Arginine	12.4	28.4
Histidine	2.3	4.0
Lysine	9.2	15.4
Reference	1	2

1. Cruft *et al.* (1957b)
2. Holbrook *et al.* (1960)

Amino Acid Analyses of the γ-Histones

Values as moles per 100 moles of amino acids

Amino Acid	Calf Thymus	Rat Liver
Aspartic Acid	6.2	13.3
Glutamic Acid	9.1	
Glycine	8.6	9.6
Alanine	12.2	12.5
Valine	6.5	6.3
Leucine	9.4	14.0
Isoleucine	4.5	
Phenylalanine	1.0	1.5
Tyrosine	3.2	2.6
Serine	6.7	4.5
Threonine	5.0	6.6
Proline	3.7	3.5
Methionine	1.0	1.2
Arginine	8.6	10.7
Histidine	2.6	2.0
Lysine	11.4	11.6
Reference	1	2

1. Cruft *et al.* (1957b)
2. Holbrook *et al.* (1960)

Amino Acid Analyses of Fractions Obtained from the Lysine-rich Histones

(Johns, 1963)

Moles per 100 moles of amino acids.

Amino Acid	Starting Material	see Fig 34 TCA precipitation (x)	(a)	(b)	IRC-50 Chromatography of fraction a a_1	a_2
Aspartic Acid	12.8	7.6	4.0	0.6	2.3	4.2
Glutamic Acid		11.3	7.5	1.0	3.2	7.9
Glycine	6.2	6.5	7.0	3.4	6.3	5.3
Alanine	21.3	12.7	20.1	29.8	25.8	20.5
Valine	4.3	5.2	4.3	1.8	4.7	4.5
Leucine Isoleucine	6.3	11.1	8.5	1.4	4.7	4.9
Phenylalanine	1.0	3.4	1.8	-	0.6	1.4
Tyrosine	-	2.0	-	-	0.5	-
Serine	5.9	6.7	7.0	3.5	5.8	5.8
Threonine	4.7	4.9	5.1	4.5	5.1	6.2
Proline	8.1	6.0	6.5	12.0	9.6	8.6
Histidine	0.3	2.1	0.8	0.2	-	0.3
Lysine	26.3	16.9	24.9	41.4	30.3	28.5
Arginine	2.8	3.8	2.6	0.4	1.3	2.1

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